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(54) Title: PROTEIN ENGINEERING OF GLUCOAMYLASE TO INCREASE pH OPTIMUM, SUBSTRATE SPECIFICITY AND THERMOSTABILITY

(57) Abstract

A fungal glucoamylase including a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair. The mutation provides increased thermal stability and reduced isomaltose formation to the enzyme. A fungal glucoamylase including a 311-314Loop mutation, wherein reduced isomaltose formation is provided by the mutation, is also provided. A fungal glucoamylase, including a mutation Ser411Ala wherein increased pH optimum and reduced isomaltose formation is provided by the mutation, is also provided. Combinations of the mutations in engineered glucoamylases are also provided as are combinations with other glucoamylase mutations that provide increased thermal stability, increased pH optimum and reduced isomaltose formation for cumulative improvements in the engineered glucoamylases.

## PROTEIN ENGINEERING OF GLUCOAMYLASE TO INCREASE
## pH OPTIMUM, SUBSTRATE SPECIFICITY AND THERMOSTABILITY

### FIELD OF THE INVENTION

5      The field of the invention relates to mutations to
produce a fungal glucoamylase enzyme that is more
selective for the production of glucose rather than the
α-1,6 linked disaccharide isomaltose, is more
thermostable, and has increased pH optimum and produces
10     increased amounts of glucose compared to wildtype
enzymes.

### BACKGROUND OF THE INVENTION

Glucoamylase (EC 3.2.1.3) is a carbohdrase. Dis-
15     covered in 1951, it is an exo-hydrolase that cleaves D-
glucose from the nonreducing ends of
maltooligosaccharides, attacking α-(1,4)-, and at a
much slower rate, α-(1,6)-glucosidic bonds.  It is one
of more than one hundred carbohydrases (EC 3.2.1) that
20     cleave O-glycosidic bonds of either α- or β-
configuration.  The functional and structural related-
ness of these enzymes is reflected in the presence of
at least three discrete regions of sequence homology
between glucoamylase and several α-amylases, α-
25     glucosidases, and transglucanosylases [Svensson, 1988],
and a similar domain structure to carbohydrases that
attack insoluble substrates [Knowles et al., 1987;
Svensson et al., 1989)].  Aspergillus awamori
glucoamylase (1,4-α-D-glucan glucohydrolase; EC
30     3.2.1.3) is one of the most important of the
glucoamylases.

Glucoamylase is primarily used in industry for the
production of high-fructose corn sweeteners in a proc-
ess that involves 1) α-amylase to hydrolyze starch to
35     maltooligosaccharides of moderate length (dextrin); 2)
Glucoamylase to hydrolyze dextrin to glucose; and 3)

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glucose isomerase to convert glucose to fructose.  Corn
sweeteners have captured over 50% of the U. S.
sweetener market, and the three enzymes used to make
them are among the enzymes made in highest volume.  In
5   addition, glucose produced by glucoamylase can be
crystallized or used in fermentation to produce organic
products such as citric acid, ascorbic acid, lysine,
glutamic acid or ethanol for beverages and fuel.
Approximately 12% of the country's corn production is
10  processed with glucoamylase.  Although glucoamylase has
been successfully used for many years, it would be a
more attractive product if it produced higher amounts
of glucose instead of disaccharides, if it were more
stable, and if it could be used in the same vessel with
15  glucose isomerase.

Glucoamylase does not give 100% yield of glucose
from dextrin because it makes various di- and
trisaccharides, especially isomaltose and
isomaltotriose, from glucose [Nikolov et al., 1989].
20  These products, formed at high substrate
concentrations, result from the ability of glucoamylase
to form $\alpha$-(1,6)-glucosidic bonds.  Glucoamylase is not
as thermostable as either $\alpha$-amylase or glucose
isomerase.  The optimum pH of GA (pH4-4.5) is lower
25  than that of $\alpha$amylase (pH5.5-6.5) and glucose isomerase
(pH7-8).  Therefore glucoamylase hydrolysis must be
done separately from the other enzymatic reactions in a
different vessel and at lower temperatures, causing
higher capital costs.

30  Glucoamylase from the filamentous fungus
Aspergillus niger is the most widely used glucoamylase,
and its biochemical properties have been extensively
characterized.  This enzyme is found mainly in two
forms, GAI (616 amino acids; referred to as AA
35  hereinafter) and GAII (512 AA), differing by the pres-
ence in GAI of a 104-AA C-terminal domain required for

adsorption to native starch granules [Svensson et al., 1982; Svensson et al., 1989]. Both forms have a catalytic domain (AA1-440) followed by a Ser/Thr-rich, highly O-glycosylated region (AA441-512) [Gunnarsson et

5     al., 1984]. The first thirty residues of this region are included in the three-dimensional structure of the enzyme [Aleshin et al., 1994; 1996; Stoffer et al., 1995]; they wrap around the catalytic domain like a belt. There is strong AA sequence homology among

10    fungal glucoamylase's in four distinct regions of the catalytic domain that correspond to the loops that form the substrate binding site [Itoh et al., 1987]. In A. niger glucoamylase these regions are AA35-59, AA104-134, AA162-196, and AA300-320. The second and third

15    regions partially or completely overlap the three regions of homology to $\alpha$-amylases [Svensson, 1988]. In addition, the raw starch binding domain (AA512-616) has high homology to similar domains from several starch-degrading enzymes [Svensson et al., 1989].

20         Kinetic analysis showed that the substrate binding site is composed of up to seven subsites [Savel'ev et al., 1982] with hydrolysis occurring between subsites 1 and 2. The $pK_a$'s of hydrolysis, 2.75 and 5.55 [Savel'ev and Firsov, 1982], suggest that carboxylic acid

25    residues at subsites 1 and 2 provide the catalytic acid and base for hydrolysis. Chemical modification experiments showed that three highly conserved residues, Asp176, Glu179, and Glu180, are protected and are in the active site, suggesting that one or more of

30    them are the possible catalytic residues [Svensson et al., 1990]. Chemical modification experiments also in-dicated that the highly conserved residue Trp120 is essential, and is located in subsite 4 [Clarke and Svensson, 1984]. Trp120 is homologous to Trp83 of

35    Aspergillus oyzae $\alpha$-amylase [Clarke and Svensson, 1984], which is also located in the active site of that

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enzyme [Matsuura *et al.*, 1984]. Site directed
mutagenesis studies have indicated that Glu179 is the
catalytic acid residue, while Glu400 is the catalytic
base residue [Frandse et al, 1994; Harris et al, 1993;
5    Sierks et al, 1990]

Glucoamylases from *A. niger* [Svensson *et al.*,
1983; Boel *et al.*, 1984] and *Aspergillus awamori*
[Nunberg *et al.*, 1984] have been cloned and sequenced,
and have identical primary structures. Innis *et al.*
10   [1985] and more recently Cole *et al.* [1988] have
developed vectors (pGAC9 and pPM18, respectively) for
glucoamylase expression in yeast, allowing convenient
manipulation and testing of glucoamylase mutants.


15                   **SUMMARY OF THE INVENTION**


According to the present invention, a fungal
glucoamylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1)
with decreased thermal inactivation (increased
20   thermostability) and reduced isomaltose formation
provided by the mutation Asn20Cys coupled with Ala27Cys
forming a disulfide bond between the two is provided.
Cumulative thermostability is also provided for GA by
including the mutation Asn20Cys coupled with Ala27Cys
25   and at least one mutation from Table 13.   An
engineered GA including Ser30Pro, Gly137Ala, and
Asn20Cys coupled with Ala27Cys provides even more
thermostability. Cumulative thermostability is also
provided for GA by including the mutation Asn20Cys
30   coupled with Ala27Cys and at least two mutations from
Table 13.

The present invention also provides a fungal
glucoamylase with reduced isomaltose formation
including an Asn20Cys coupled with Ala27Cys mutation
35   (S-S mutation) and at least one mutation selected from
Table 14. In an embodiment Asn20Cys coupled with

Ala27Cys mutation and a 311-314Loop (also referred to as 300Loop) mutation are included in an engineered GA. In a further preferred embodiment the engineered glucoamylase with reduced isomaltose formation includes

5    Asn20Cys coupled with Ala27Cys mutations Ser30Pro and Gly137Ala.

The present invention also provides engineered fungal glucoamylase including a 311-314Loop mutation whereby reduced isomaltose formation is provided by the

10   mutation. In a further embodiment fungal glucoamylase including a 311-314Loop mutation and at least one mutation from Table 14 are prepared whereby cumulative reduced isomaltose formation is provided by the additional mutation.

15   The present invention provides a fungal glucoamylase including a mutation Ser411Ala whereby increased pH optimum and reduced isomaltose formation is provided by the mutation. In an embodiment the Ser411Ala mutation is combined with at least one

20   mutation from Table 15 whereby cumulative increased pH optimum is provided by the mutations. In an embodiment the Ser411Ala mutation is combined with at least one mutation from Table 14 whereby cumulative reduced isomaltose formation is provided by the mutations.

25   In a further embodiment an engineered fungal glucoamylase includes a mutation Ser411Ala and a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair whereby increased thermal stability, increased pH

30   optimum and reduced isomaltose formation are provided by the mutations.

In a still further embodiment a fungal glucoamylase is engineered to include a Ser411Ala mutation and a mutation pair Asn20Cys coupled with

35   Ala27Cys forming a disulfide bond between the two members of the pair and a 311-314Loop mutation whereby

increased thermal stability, increased pH optimum and
reduced isomaltose formation are provided by the
mutations.

5      The present invention provides a method to obtain
a fungal glucoamylase with reduced isomaltose formation
by designing mutations to decrease the $\alpha$-(1,6)-
glucosidic linkage affinity of GA.

The present invention also provides a method to
obtain a fungal glucoamylase with decreased thermal
10     inactivation by designing mutations to decrease the
enzyme's conformational entropy of unfolding and/or
increase stability of $\alpha$-helices, increase disulfide
bonds, hydrogen bonding, electrostatic interactions,
hydrophic interactions, Vanderwalls interactions and
15     packing compactness.

The present invention also provides a fungal
glucoamylase with increased pH optimum including
changing the polarity, charge distribution and hydrogen
bonding in the microenvironment of the catalytic base
20     Glu400.

The present invention also provides a method of
genetically engineering glucoamylase carrying at least
two cumulatively additive mutations.  Individual
mutations are generated by site-directed mutagenesis.
25     These individual mutations are screened and those
selected which show increased pH optimum and which show
decreased irreversible thermal inactivation rates or
reduced isomaltose formation.  Site directed
mutagenesis is then performed to produce enzymes
30     carrying at least two of the isolated selected
mutations.  Finally the engineered enzymes are screened
for cumulatively additive effects of the mutations on
thermal stabilizing or reduced isomaltose formation by
the produced enzymes carrying at least two of the
35     isolated selected mutations.  Alternatively, the
engineered enzyme is screened for cumulatively additive

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effects of both of the mutations on pH optimum, thermostability and/or reduced isomaltose formation by the produced enzymes carrying at least two of the isolated selected mutations.

5          Vectors for each of the mutations and mutation combinations are also provided by the present invention as well as host cells transformed by the vectors.

## DESCRIPTION OF THE DRAWINGS

10

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the 15 accompanying drawings wherein:

FIGURE 1 is a graph showing the relationship between temperature and *kd* for wild-type (●) and proline substituted mutant GA's: S30P (■), D345P (v), E408P (◊) in Example 1.

20          FIGURE 2 is a graph showing effect of temperature on first-order thermoinactivation rate coefficients of wild-type (O), A27C (●), N20C (v), A27C/N20C (▼), A471C/T72C (□), A27C/N20C/G137A (■), A27C/N20C/S436P (◊) AND G137A/S436P (♦) glucoamylases measured in pH 25 4.5 buffer.

FIGURE 3 is a graph showing initial reaction rates of wild-type (O), A27C/N20C (●), A471C/T72C (v) and A29C/N20C/G137A (▼) glucoamylases with 4% maltose in 0.05 M sodium acetate (pH 4.5) as substrate at 30 temperatures from 60°C to 76°C.

FIGURE 4 is a graph showing the effect of temperature on the activity of wildtype and mutant GA. Error bars represent the standard deviation from three assays. Wildtype (●), S30P/G137A (□), S-S/S30P/G137A 35 (▲).

FIGURE 5A-C are graphs showing the effect of

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temperature on irreversible thermal inactivation rate
coefficients of wildtype and mutant GA.  Fig. 5A
Wildtype (●), S30P (■), G137A (▲), S30P/G137A (□); Fig.
5B Wildtype (●), S30P (■), S-S (hexagon), S-S/S30P

5    (filed circle with empty center); Fig. 5C Wildtype (●),
S30P/G137A (□), S-S/S30P (filed circle with empty
center), S-S/S30P/G137A (▲).

FIGURE 6A-B are graphs showing saccharification of
28% (w/v) Maltrin M100 by wildtype (●), S30P/G137A (□)

10   and S-S/S30P/G137A (▲).

FIGURE 7 is a graph showing the 30% DE 10
maltodextrin saccharification of wildtype (♦) and
mutant glycoamylases: 300Loop (■), S30P/G137A (▲), S-S
(●), S30P/G137A/300Loop (x), S-S/300Loop (△), at 55°C,

15   enzyme concentration was 166.67 μg/mL in each reaction.

FIGURE 8 is a graph showing production of
isomaltose by wildtype (●) and mutant glucoamylases:
Y116W (■), Y175F (▲), R241K (▼), S411A (♦), S411G
(hexagon), during glucose condensation at 55°C with 30%

20   (w/v) D-glucose in 0.05M sodium acetate buffer at pH4.4
with 0.02% sodium azide for 12 days.

FIGURE 9 is a graph showing the production of
glucose by wildtype (●) and mutant glucoamylases: Y116W
(■), Y175F (▲), R241K (▼), S411A (♦), S411G (hexagon),

25   during hydrolysis of DE 10 maltodextrin at 55°C with
28% (w/v) maltodextrin in 0.05M sodium acetate buffer
at pH4.4 with 0.02% sodium azide for 12 days.

FIGURE 10 is a graph showing the initial rates of
glucose production by wildtype (●) and S411A (■)

30   glucoamylases during DE 10 maltodextrin hydrolysis at
different pH values.  Hydrolysis was performed at 36°C
with 28% (w/v) maltodextrin in 25mM citrate-phosphate
buffer at indicated pHs with 0.02% sodium azide for 4
days.

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## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides mutations for
increased thermal stability, increased pH optimum and
5    reduced isomaltose formation in the glucoamylase from
fungal species which may provide increased glucose
yields compared to wildtype glucoamylase. Predicted
structure and known sequences of glucoamylase are
conserved among the fungal species [Coutino et al,
10   1994]. As an exemplar *Aspergillus awamori* glucoamylase
(1,4-α-D-glucan glucohydrolase; EC 3.2.1.3; referred to
as GA herein; SEQ ID No:1) is used, but any other
fungal species including *Aspergillus* species
glucoamylase can be used. The numbering of the
15   glucoamylase amino acids herein is based on the
sequence of the exemplar *Aspergillus awamori*.
Equivalent amino acid residue numbers are determined
differently for different fungal species as is known in
the art [Coutino et al., 1994].
20       The present invention provides a fungal
glucoamylase with decreased thermal inactivation
(increased thermostability) and decreased isomaltose
formation provided by engineering the inclusion of a
mutation pair Asn20Cys coupled with Ala27Cys which
25   forms a disulfide bond between them (this mutation is
abbreviated as Asn20Cys/Ala27Cys or S-S). Additional
mutations providing decreased thermal inactivation are
set forth in Summary Table 13.
         Cumulative thermostability is also provided for GA
30   by including at least two of the mutations in the
enzyme as for example including mutations Ser30Pro and
Gly137Ala. Another example is to engineer S-S with
Asn20Cys/Ala27Cys in the enzyme or to pair Gly137Ala
with S-S. Further, combinations of the individual
35   mutations set forth in Table 13, particularly with S-S
coupled with Ser30Pro also provide cumulative

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thermostability.  In general two mutation combinations
are made but triple mutations can also be constructed.
As for example, an engineered GA including the three
mutations: Ser30Pro, Gly137Ala, and Asn20Cys/Ala27Cys
5   provides even more thermostability.

By Asn20Cys coupled with Ala27Cys is meant a pair
of mutations which is abbreviated as "S-S" or
Asn20Cys/Ala27Cys and between which is formed a
disulfide bond as described herein in the Examples.  In
10  general, this is referred to as a single mutation since
both are required to form the disulfide bond.

By cumulative is generally meant the additive (or
nearly additive) effects of two or more mutations on
the parameter of enzyme activity being measured.
15      The present invention also provides a fungal
glucoamylase with reduced isomaltose formation and
increased glucose yield including the Asn20Cys/Ala27Cys
mutation (S-S mutation) and at least one mutation
selected from Table 14.  In an embodiment the
20  Asn20Cys/Ala27Cys mutation and the 311-314Loop
(300Loop) are included in GA.  In a further preferred
embodiment the engineered glucoamylase with reduced
isomaltose formation includes Asn20Cys/Ala27Cys and
with mutations Ser30Pro and Gly137Ala.
25      In an embodiment a glucoamylase with the 311-114
loop mutation is constructed to provide reduced
isomaltose formation.  By the 311-314Loop mutation is
meant an insertional GA mutant with the sequence
Tyr311-Tyr312-Asn313-Gly314→Tyr311-Asn-Gly-Asn-Gly-Asn-
30  Ser-Gln-Gly314 (311-314 Loop; SEQ ID No:2).

The present invention provides a fungal
glucoamylase including a Ser411Ala mutation whereby
increased pH optimum and reduced isomaltose formation
is provided by the mutation.  In an embodiment the
35  Ser411Ala mutation is combined with at least one
mutation from Table 15 whereby cumulative increased pH

optimum is provided by the combined mutations.  In a
further embodiment the Ser411Ala mutation is combined
with at least one mutation from Table 14 whereby
cumulative reduced isomaltose formation is provided by
5    the mutations.

In a further embodiment an engineered fungal
glucoamylase includes a Ser411Ala mutation and the
mutation pair Asn20Cys/Ala27Cys forming a disulfide
bond between them whereby increased thermal stability,
10   increased pH optimum and reduced isomaltose formation
are provided by the mutations.

In a still further embodiment a fungal
glucoamylase including a Ser411Ala mutation and a
mutation pair Asn20Cys coupled with Ala27Cys forming a
15   disulfide bond between the two members of the pair and
a 311-314Loop mutation whereby increased thermal
stability, increased pH optimum and reduced isomaltose
formation are provided by the combination of mutations.

Mutations are indicated by the amino acid being
20   replaced followed by the residue number followed by the
replacing amino acid.  Amino acids are abbreviated
either with the three letter code or single letter
code.  Mutations are generated using site directed
mutagenesis as is known in the art.  The sequence and
25   residue number are from the Wildtype (WT) or nonmutant
enzyme.  Biochemical characterization is performed as
described herein below and in the Examples.  The
Examples provide exemplars of the analysis for an
individual mutation to determine it's characteristics
30   and provide exemplars of analysis for combinations of
mutations to determine if the combination provides a
cumulative effects.

By increased thermostability (or decreased thermal
inactivation) is meant that at temperatures between
35   65°C and 77.5°C the mutants are irreversibly
inactivated at a decreased rate compared to wildtype.

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The present invention provides a method to obtain
fungal glucoamylases with decreased thermal
inactivation by designing mutations to decrease the
rate of irreversible thermal inactivation at
5      temperatures between 65°C and 77.5°C compared to
wildtype. This is accomplished by designing
glucoamylases with decreased thermal inactivation by
designing mutations to decrease the enzyme's
conformational entropy of unfolding and/or increase
10     stability of α-helices, increase disulfide bonds,
hydrogen bonding, electrostatic interactions, hydrophic
interactions, Vanderwalls interactions and packing
compactness.

Basic mechanisms underlying protein
15     thermostability and factors influencing reversible and
irreversible thermal inactivation have been studied
extensively [Argos et al., 1979; Klibanov, 1983;
Wasserman, 1984; Ahern and Klibanov, 1985]. Factors
involved in stabilizing proteins at high temperatures
20     include 1) disulfide bonds, 2) noncovalent bonds such
as salt bridges, hydrogen bonding, and hydrophobic
interactions, and 3) conformational rigidity [Nosoh and
Sekiguchi, 1988]. The causes of irreversible
inactivation at high temperatures include 1)
25     aggregation, 2) the formation of incorrect structures,
3) the destruction of disulfide bonds, 4) deamidation
(especially of Asn at Asn-Gly sequences), and 5)
cleavage of Asp-X peptide linkages. It is apparent
that replacement of even one residue can make a large
30     difference in protein thermostability [Matsumura and
Aiba, 1985], due to the small increases in free energy
(20-30 kJ/mol) usually required to stabilize protein
tertiary structures [Nosoh and Sekiguchi, 1988].
Genetic engineering to increase thermostability (or to
35     decrease irreversible thermoinactivation) of enzymes
has been successful in several cases [Perry and Wetzel,

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1984; Imanaka *et al.*, 1986; Ahearn *et al.*, 1987].
However, the mechanisms that govern thermostability are
not fully understood, so that amino acid (AA)
replacements that promote thermostability are not

5     accurately predicted [Leatherbarrow and Fersht, 1986;
Nosoh and Sekiguchi, 1988; Pakula and Sauer, 1989].
The method of the present invention allows for more
accurate prediction.

By increased pH optimum is meant that the enzyme

10    is functional at a higher pH, above that of wildtype.

The present invention also provides a method to
design a fungal glucoamylase with increased pH optimum
by changing the polarity, charge distribution and
hydrogen bonding in the microenvironment of the

15    catalytic base Glu400. For example, mutants S411G and
S411A were designed to remove the hydrogen bond between
Ser411 and Glu400 (see Example 8).

By increased selectivity is meant that there is
decreased isomaltose formation due to decrease in the

20    production of undesirable $\alpha$-(1,6)-linked byproducts
(reversion products) at high glucose concentrations
[Lee *et al.*, 1976]. As described above, GA hydrolyzes
and synthesizes both $\alpha$-(1,4) and $\alpha$-(1,6) glucosidic
bonds. Increasing selectivity indicates that the

25    enzyme synthesizes $\alpha$1,6 linked products at a lower rate
than wildtype as shown by reduced levels of isomaltose
formation in condensation reactions with 30% glucose as
a substrate compared to wildtype GA. Additionally,
improved selectivity may result in increased glucose

30    yields in saccharification reactions using 28% DE 10
maltodextran as a substrate.

The present invention provides a method to obtain
a fungal glucoamylase with reduced isomaltose formation
by designing mutations to decrease the $\alpha$-(1,6)-

35    glucosidic linkage affinity of GA. That is mutations
are designed in the active site to reduce isomaltose

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formation due to glucose condensation. The mutations
are designed to have decreased ability to synthesize
isomaltose while maintaining at least partial wildtype
ability to digest α1-4 linked substrates resulting in a
5    lower ratio of isomaltose formation to glucose
formation than wildtype. These mutations are made at
positions that are not completely conserved based on
homology analysis.

Kinetic studies have indicated that there are five
10   to seven glucosyl binding subsites, and the catalytic
site is located between subsites 1 and 2 [Hiromi et
al., 1973, Hiromi et al., 1983, Meagher et al., 1989,
Tanaka et al., 1983]. The solved three-dimensional
structure of the catalytic domain of glucoamylase from
15   *Aspergillus awamori var X100*, which has about 95%
homology with the corresponding regions of GAs from
*Aspergillus awamori* and *Aspergillus niger* [Coutinho &
Reilly, 1994], contains thirteen alpha-helices, twelve
of which are arranged in pairs forming an alpha/alpha
20   barrel [Aleshin et al., 1992, Aleshin et al., 1994].
The active site is located in the cavity of the barrel
center. In addition, homology analysis of thirteen
amino acid sequences of glucoamylases showed that five
conserved regions define the active site [Coutinho &
25   Reilly, 1994]. The mechanism of GA catalysis involves
two carboxyl groups [Hiromi et al., 1966], Glu179 and
Glu400 (in *Aspergillus awamori* or *Aspergillus niger*)
[Frandsen et al., 1994, Harris et al., 1993, Sierks et
al., 1990]. Glu179 protonates the oxygen in the
30   glycosidic linkage, acting as general acid acatalyst,
and Glu400 activates water (Wat500) for nucleophilic
attack at carbon C-1, acting as a general base catalyst
[Frandsen et al., 1994]. The crystal structures of
glucoamylase complexed with the pseudotetrasaccharides
35   (acarbose and D-gluco-dihydroacarbose), showed that
there are two different binding conformers, pH 4-type

and pH 6-type, for pseudotetrasaccharides at pH 4
[Aleshin et al., 1996, Stoffer et al., 1995]. Binding
of the first two sugar residues of the
pseudotetrasaccharides is the same, but there is an

5    extraordinary variation in binding of the third and
fourth sugar residues of the pseudotetrasaccharides
[Stoffer et al., 1995].

    The substrate specificity of an enzyme is
determined by its ability to form a stable complex with

10   a ligand in both the ground state and the transition
state. The stability of the enzyme-ligand complex is
affected by steric constraints, hydrogen bonding, van
der Waal's and electrostatic forces, and hydrophobic
contacts [see generally Fersht, 1985 Enzyme Structure

15   and Mechanism, 2nd edition, Freeman, San Francisco].
Site-directed mutagenesis was used to construct
mutations at residues 119 and 121 to alter the hydrogen
bonding between enzyme and substrate. Atom OG of
Ser119 hydrogen bonds to the 3-OH of the fourth sugar

20   residue of pseudo-tetrasaccharides only in the pH 6-
type conformer, whereas the amide nitrogen of Gly121
hydrogen bonds to the 6-OH of the third sugar residue
in both pH 4-type and pH 6-type conformers. These
mutations are designed to change substrate specificity

25   (decrease alpha-1,6 condensation reactions) while
maintaining wild-type ability to hydrolyze alpha-1,4
linked substrates. Ser119 is not conserved and is
replaced by Ala, Pro and Glu in other GAs. Mutant
S119E was designed to strengthen the hydrogen bond

30   between the enzyme and the fourth sugar residue of the
substrate to stabilize the pH 6-type conformer, and to
bring a negative charge near subsite 4 in order to
increase electrostatic interactions in active site.
Mutant S119G was designed to remove the same hydrogen

35   bond in order to destabilize the pH 6-type conformer.
Mutant S119W was designed to remove the same hydrogen

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bond and to increase the hydrophobic interactions
between the enzyme and the pH 6-type conformer.  Gly121
is highly conserved in all glucoamylase sequences
except in *Clostridium sp. G005* GA, which has high $\alpha$-1,6
5    activity and in which Gly is replaced by Thr.  Since
the $\phi$ and $\psi$ angles of Gly121 would allow an alanine in
this position without causing a conformation
distortion, G121A was designed to introduce a Beta-
carbon at position 121 to displace the 6-OH group of
10   the third sugar residue from its hydrogen bonding
position.  In addition, the double mutant G121A/S411G
was designed to investigate additivity of the two
substrate specificity mutations.  S411G is shown herein
to reduce the ratio of initial rates of isomaltose
15   production (from glucose condensation reactions) to
that of glucose production (from the hydrolysis of DE
10 maltodextrin).

The following provide further examples of the
strategies used for the design of mutations having
20   increased selectivity.

300Loop mutation:  According to the amino acid sequence
homology study [Countinho and Reilly, 1994], it was
found that GAs from *Rhizopus* and some other fungal
families have a longer amino acid sequence and form a
25   larger loop or cavity in the S4 conserved region
compared to *A. niger* or *A. awamori* GAs.  Since single
mutation events alone are unlikely to bring about
substantial increase in the specificity of bond
hydrolysis or synthesis, an insertional mutant GA was
30   designed, designated 300Loop or 311-314Loop (SEQ ID
NO:2), and the inserted seven amino acids were adapted
from *Rhizopus* GA because *Rhizopus* GA was the first
enzyme to which the subsite theory was successfully
applied [Himori et al., 1973].  The 300Loop mutation
35   was designed to decrease the $\alpha$-(1,6)-glucosidic
affinity by introducing a larger loop into the S4

conserved region.

Tyr175Phe: Tyr175 is within the third conserved region.
The nearest distance between Tyr175 and the fourth
residue of inhibitor D-*gluco*-dihydroacarbose is 4.06 Å

5        [Stoffer et al., 1995]. Tyr175 is replaced by Phe or
Gln in several other glucoamylases. Changing Tyr175 to
Phe was designed to increase the hydrophobic
interaction between enzyme and substrate.

Gly121Ala: Gly121 is highly conserved in all

10       glucoamylase sequences except in *Clostridium sp. G005*
GA, which has high $\alpha$-1,6 activity and in which Gly is
replaced by Thr. Since the $\phi$ and $\psi$ of Gly121 would
allow an alanine in this position without causing a
conformation distortion, G121A was designed to

15       introduce a $\beta$-carbon at position 121 to displace the 6-
OH group of the third sugar residue from its hydrogen
bonding position.

Gly121Ala with S411G (generally indicated as
G121A/S411G): The double mutant was designed to

20       investigate additivity (cumulative) of the two
substrate specificity mutations. S411G reduces the
ratio of initial rates of isomaltose production (from
glucose condensation reactions, see Examples) to that
of glucose production (from the hydrolysis of

25       maltodextrin 10).

        The present invention provides a method of
engineering mutations for fungal glucoamylase and then
preparing engineered enzymes carrying cumulatively
additive mutations. The initial step is to generate

30       individual mutations by site directed mutagenesis and
screen the individual mutations as described in the
Examples. Those individual mutations which show
decreased irreversible thermal inactivation rates or
reduced isomaltose formation or increased pH optimum

35       are then selected for combinational analysis. In
general mutations are selected which have at least

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wildtype reaction rates.

Mutations are combined by site-directed
mutagenesis to determine if their effects are additive
as is discussed herein in the Examples.  Site directed

5    mutagenesis to produce enzymes carrying at least two of
the isolated selected mutations is performed as is
known in the art.  These engineered enzymes are then
screened for cumulatively additive effects on thermal
stabilizing, pH optimum or reduced isomaltose

10   formation.  Alternatively the engineered enzymes
carrying cumulative mutations are screened for
cumulative effects on two or more of the parameters.

For biochemical characterization of the mutants,
GA is purified from culture supernatants of 15-L batch

15   fermentations by ultrafiltration, DEAE-Sephadex column
chromatography, and column affinity chromatography
using the potent inhibitor acarbose attached to a
support [Sierks et al., 1989].  Purities of the
resulting preparations are tested by standard

20   techniques such as SDS-polyacrylamide gel
electrophoresis and isoelectric focusing with narrow-
band ampholytes.  Protein are measured by absorbance at
280 nm or by Bradford's method [1976].  GA activity is
measured by a glucose oxidase/o-dianisidine assay

25   (Sigma)..

Selectivity is determined by any method known in
the art but preferably by measuring the initial rate of
isomaltose formation from 30% (w/v) glucose
condensation reactions at pH 4.4 and 55°C in 0.05M

30   sodium acetate buffer and then by measuring the initial
rage of glucose formation in 30% (w/v) DE 10
maltodextran hydrolysis reactions at pH 4.4 and 55°C
0.05M sodium acetate buffer.  From the resulting
initial rates, the ratio of isomaltose formation to

35   glucose formation is calculated.

Thermostability is measured as is known in the art

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but preferably by incubating the enzyme at selected
temperatures between 65°C and 77.5°C at 2.5°C intervals
followed by activity analysis at 35°C using 4% maltose
as substrate.  When first-order decay is observed, as
5    with WT GA, decay rate coefficients are determined.
Activation energies for decay are calculated from the
rate coefficients at different temperatures.

      pH optimum is measured as is known in the art but
preferably at 45°C at 16 pH values, ranging for 2.2 to
10   7.0 using 0.025 M citrate-phosphate buffer with maltose
or maltoheptaose as substrate.

      Saccharification is measured as described in the
Examples.  Briefly, glucoamylase is incubated with DE
10 maltodextran as substrate in 0.05M sodium acetate
15   buffer at pH 4.4 at 55°C.  Samples are taken at various
times from 0.5 to 288 hours and the production of
glucose determined.

      The present invention provides vectors
comprising an expression control sequence operatively
20   linked to the nucleic acid sequence of the various
mutant sequences disclosed herein, combinations of
mutations and portions thereof.  The present invention
further provides host cells, selected from suitable
eucaryotic and procaryotic cells, which are transformed
25   with these vectors.

      Vectors can be constructed containing the cDNA of
the present invention by those skilled in the art and
should contain all expression elements necessary to
achieve the desired transcription of the sequences.
30   Other beneficial characteristics can also be contained
within the vectors such as mechanisms for recovery of
the nucleic acids in a different form.  Examples are
provided herein.  Phagemids are a specific example of
such beneficial vectors because they can be used either
35   as plasmids or as bacteriophage vectors.  Examples of
other vectors include viruses such as bacteriophages,

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baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of

5  ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art (calcium phosphate transfection;

10  electroporation; lipofection; protoplast fusion; polybrene transfection; ballistic DNA delivery; lithium acetate or CaCl transformation). The host cell can be any eucaryotic and procaryotic cells, which can be transformed with the vector and which will support the

15  production of the enzyme.

The above discussion provides a factual basis for thermostable and selective mutants of fungal glucoamylase as well as methods of designing the mutations and screening for the cumulative effect of

20  the mutations and vectors containing the mutations. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

25                             **EXAMPLES**

**General methods in molecular biology**: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory*

30  *Manual*, Cold Springs Harbor Laboratory, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and Rose, et al. *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring

35  Harbor Laboratory Press, Cold Spring Harbor, NY (1990). Polymerase chain reaction (PCR) was carried out

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generally as in *PCR Protocols: A Guide To Methods And*
*Applications*, Academic Press, San Diego, CA (1990).
Oligonucleotides are synthesized as is known in the
art.  For example, an Applied Biosystems 380B DNA
5    synthesizer can be used.

    **Materials**:  *S. cerevisiae* C468 ($\alpha$ *leu2-3 leu 2-112*
*his 3-11 his 3-15 mal*) and the plasmid YEpPM18 were
gifts from Cetus.  Acarbose was a gift from Miles
Laboratories.  All restriction enzymes were purchased
10   from Promega as well as T4 DNA ligase and pGEM-7Z(+),
an *E. coli* phagemid vector, were from Promega.  Maltose
($G_2$), maltotriose ($G_3$), maltotetraose ($G_4$), malto-
pentaose ($G_5$), maltohexaose ($G_6$), maltoheptaose ($G_7$),
glucose oxidase, peroxidase, and $\alpha$-naphthol were from
15   Sigma.  Isomaltose ($iG_2$) was purchased from TCI America.
DE 10 Maltodextrin with the average degree of
polymerizations (DP) of 10, 6, and 4, respectively,
were from Grain Processing Corporation. High-
performance thin-layer chromatographic (HPTLC) plates
20   (LHPK silica gel 60 Å, 20 x 10 cm) were from Whatman.

    **Site-directed mutagenesis**:  Site-directed
mutagenesis was performed according to the Muta-Gene
phagemid *in vitro* mutagenesis kit from Bio-Rad which is
based on the method of Kunkel *et al* [1985].  A 1.7 kb
25   XhoI→BamHI DNA fragment coding for the glucoamylase
catalytic domain was cloned into a pBluescript II KS(+)
vector from Stratagene.  Oligonucleotides used as
mutagenic primers are provided with the specific
Example.  The presence of the individual mutations was
30   confirmed by sequencing and each mutated GA gene
fragment was subcloned into YepPM18 [Cole, *et al.*,
1988] and transformed into *S. cerevisiae*.

    **Enzyme production and purification**: Wild-type (WT)
and mutant enzymes are produced by growing yeast at
35   30°C in 5.3 L SD + His media for 72 hours at pH 4.5 in
a 5.0 L fermentor.  After 48 hours, 100g of dextrose

and 22g of $(NH_4)_2SO_4$ in 300ml $H_2O$ is added as a
supplement. Following growth, the culture is
centrifuged to remove yeast cells, the supernatant is
concentrated by ultrafiltration, diafiltered against
5      0.5 M NaCl/0.1 M NaOAc, pH 4.5 and purified by
acarbose-sepharose affinity chromatography. GA is
eluted with 1.7 M Tris-Cl, pH 7.6, dialyzed against $H_2O$,
further concentrated by ultrafiltration and diafiltered
against 0.05 M NaOAc buffer, pH 4.5. The protein
10     concentration is determined according to the Pierce
bicinchoninic acid protein assay [Smith et al., 1985]
using bovine serum albumin as a standard.

      **Enzyme activity assays**: Enzyme activities were
determined at 50°C using 4% maltose in 0.05 M NaOAc
15     buffer pH 4.5 as substrate. One international unit
(IU) of enzyme activity was defined as the amount of
enzyme required to produce 1 $\mu$mol/min glucose at assay
conditions. Following mixing enzyme with substrate,
six 100 $\mu$l samples were removed at seven minute
20     intervals over 42 minutes, the reaction stopped with 40
$\mu$l of 4.0 M Tris-Cl, pH 7.0 and the glucose
concentration was determined according to the Sigma
peroxidase-glucose oxidase/o dianisidine glucose assay
kit.

25           **Irreversible thermal inactivation**: Duplicate
aliquotes of 40 $\mu$g/ml of purified wild-type and mutant
enzymes were subjected to inactivation at six or more
temperatures between 65° and 80°C at intervals of
2.5°C. Samples were removed at six different time
30     points, immediately placed on ice and stored at 4°C for
24 hours. The residual activity of the inactivated
samples along with a corresponding sample which had not
been subjected to thermal inactivation, was determined
as described above but at 35°C.

35           **pH dependence of glucoamylase activity**: pH
dependence of glucoamylase activity was measured at

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45°C at 16 different pH values, ranging from 2.2 to
7.0, using 0.025 M citrate-phosphate buffer [McIlvane,
1921] with maltose or maltoheptaose as substrate.  The
ionic strength of the citrate-phosphate buffer was
5    maintained at 0.1 by adding potassium chloride.  The pK
values of free enzyme and enzyme-substrate complex were
measured at substrate concentrations (i) smaller than
0.2 $K_m$, so that the initial rate ($v$) was proportional to
$k_{cat}/K_m$, and (ii) higher than 10 $K_m$, so that the initial
10   rate ($v$) was proportional to $k_{cat}$ [Sierks & Svensson,
1994, see also Whitaker (1994) Principle of enzymology
for the food sciences, 2$^{nd}$ edition, Marcel Dekker, NY].
The pK values of two catalytic groups of free enzyme
and enzyme-substrate complex were calculated by fitting
15   the initial rates as a function of pH values to the
equation log $Y$ = log $[C/(1+H/K_1+K_2/H]$ by using the
software of Enzfitter.  $Y$ is the observed value of the
parameter of interest (i.e. $k_{cat}/K_m$ or $k_{cat}$) measured at
different pH values, $C$ is the pH independent value of $Y$
20   (i.e. the maximal value of $k_{cat}/K_m$ or $k_{cat}$), $H$ is the
concentration of hydrogen ion, $K_1$ and $K_2$ are
dissociation constants of catalytic groups of enzyme.
When the values of apparent pK$_1$ and pK$_2$ were separated
by less than 3 pH units, the pK values were adjusted by
25   equations $(H^+)_1$ + $(H^+)_2$ = $K_1$ + $4(H^+)_{opt}$ and $(H^+)_{opt}$ = $\sqrt{K_1 K_2}$
[Whitaker, 1994].  The concentration of hydrogen ion at
the optimum pH, $(H^+)_{opt}$, was calculated from pHopt which
is equal to the average of apparent pK$_1$ and pK$_2$.  $(H^+)_1$
and $(H^+)_2$ (apparent $K_1$ and $K_2$) correspond to the
30   concentrations of hydrogen ion when the pH values are
equal to apparent pK$_1$ and pK$_2$, respectively.

**The hydrolysis of DE 10 maltodextrin
(Saccharification)**: Hydrolysis was performed at 35°C
and/or 55°C (as indicated in the text) with 28% (w/v)
35   DE 10 maltodextrin as substrate in 0.05 M sodium
acetate buffer at pH 4.4 with the addition of 0.02%

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sodium azide, used to inhibit microbial growth in the
reaction mixtures.  The enzyme concentration was 2.64
μM for both wild-type and mutant GAs.  Samples were
taken at various times (from 0.5 to 288 hours) and the
5    reactions were stopped by adding samples to the same
volume of 1 M Tris-HCl buffer at pH 7.0, since Tris is
a known inhibitor of glucoamylase [Clarke & Svensson,
1984].  The production of glucose was determined by the
glucose oxidase method [Rabbo & Terkildsen, 1960].
10   Initial rates of glucose production were determined by
fitting the experimental data to the equation $c =$
$At/(1+Bt)$, where $c$ is the product concentration, $t$ is
time, and $A$ (the initial rate) and $B$ are obtained from
the nonlinear regression.  At 55°C, only the time
15   points before 70 hours were used for the calculations,
since the glucose production by that time had already
declined for wild-type GA.

**Glucose condensations reactions**: Glucose
condensation reactions were performed at 35°C and 55°C
20   with 30% (w/v) D-glucose as substrate in 0.05 M acetate
buffer at pH 4.4 for 12 days with the addition of 0.02%
sodium azide, used to inhibit microbial growth in the
reaction mixtures.  The enzyme concentration was 2.64
μM for both wild-type and mutant GAs.  Samples were
25   taken at various times and the reactions were stopped
by adding samples to the same volume of 1 M Tris-HCl
buffer at pH 7.0.  High Performance Thin Layer
Chromatography (HPTLC) and Imaging Densitometry were
used to determine the production of isomaltose by a
30   method modified from that described by Robyt et al.
[Robyt and Mukerjea, 1994].  One microliter of
variously diluted samples and six different
concentrations of standard (containing glucose, maltose
and isomaltose) were applied to the HPTLC plates.  The
35   developing solvent system contained acetonitrile, ethyl
acetate, 1-propanol and water in the volume proportions

of 85:20:50:40.  Only one ascent was used to develop
the carbohydrate separation on HPTLC plates.  After
development, the plates were air-dried, dipped into an
EtOH solution containing 0.3% (w/v) alpha-naphthol and
5  5% (v/v) $H_2SO_4$, air-dried again, and incubated
approximately 10 min at 120°C to visualize the
carbohydrates.  Densities of the isomaltose spots on
HPTLC plates were quantified by Imaging Densitometry
(Bio-Rad, Model GS-670), using Molecular Analyst
10  software (Bio-Rad).  The experimental data were fitted
to the equation $c = At/(1+Bt)$, described above for the
hydrolysis of DE 10 maltodextrin, to obtain the initial
rates of isomaltose production.

15

## EXAMPLE 1

### STABILIZATION OF *ASPERGILLUS AWAMORI* GLUCOAMYLASE
### BY PROLINE SUBSTITUTION

20       The following example is an exemplar of the
methods and procedures that are used in the analysis of
an individual mutation of a glucoamylase.  To
investigate the mechanisms governing *Aspergillus
awamori* glucoamylase thermal stability, three proline
25  substitution mutations were constructed.  These
mutations were predicted to increase GA stability by
decreasing the enzyme's conformational entropy of
unfolding.
       *Aspergillus awamori* glucoamylase ($\alpha$-1,4-D-glucan
30  glucohydrolase, EC 3.2.1.3; GA) is an enzyme which
catalyses the release of $\beta$-glucose from the non-
reducing ends of starch and related oligosaccharides.
GA is used in, and defines the rate limiting step of,
the commercial conversion of starch to high glucose
35  syrups which may be converted to fructose syrups by
glucose isomerase, or used in fermentations to produce

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ethanol.  GA is used industrially at 55°-60°C; at
higher temperatures the enzyme is rapidly and
irreversible inactivated.  Therefore, a GA
variant with increased thermostability would be
5     advantageous industrially to decrease reaction times
and/or to increase solids concentrations.

Previous work has shown that the natural stability
of oligo 1,6-glucosidase [Suzuki et al., 1987] and
pullulanase [Suzuki et al., 1991] can be positively
10    correlated to the mole percent proline present in the
protein, and a general rule for protein stability has
been proposed [Suzuki, 1989].  This work has been
extended to show that bacteriophage T4 lysozyme
[Matthews et al., 1987] and *Bacillus cereus* ATCC 7064
15    oligo 1,6 glucosidase [Watanabe et al, 1994] can be
stabilized by engineering proline into selected sites
thereby decreasing the protein's conformational entropy
of unfolding.

Three sites (Ser30, Asp345 and Glu408→Pro) were
20    selected for proline substitution based on structural
and evolutionary considerations.  Mutations at these
sites were constructed using the cloned *A. awamori* gene
[Innis et al, 1985] and the proteins were expressed in
*Saccharomyces cerevisiae* [Cole, et al, 1988].  The
25    stability of the mutant proteins was measured by their
resistance to irreversible thermal inactivation at
various temperatures.  As shown herein, the Ser30→Pro
mutation increased.  However, unexpectedly the
Glu408→Pro mutation decreased and the Asp345→Pro
30    mutation did not significantly alter GA stability.

**Site-directed mutagenesis**:  Site-directed
mutagenesis was performed as described herein above.
The following oligonucleotides were used as mutagenic
primers:  CAGAGTCCGCGCCCG<u>G</u>CACCCAAGCACCGTC (Ser30→Pro)
35    (SEQ ID No:3), AAGTCCAGCGACACA<u>GG</u>TGTGACCTCCAACGAC
(Asp345→Pro) (SEQ ID No:4) and CGAGCGGAAAGCTGC

<u>GGG</u>CCATCAGACTTGTC (Glu408→Pro) (SEQ ID No:5).

**Selection of sites for proline substitution:**

Based on the nearly identical catalytic domain of *A. awamori* var *X100* GA whose structure is known [Aleshin
5  et al, 1992] three sites for substitution were chosen,
which met the following criteria:  1) Ramachandran
($\phi,\psi$) angles were within allowed values for proline
[Ramachandran et al., 1963].  For this work the $\phi$ and $\psi$
angles at the substituted site were restrained to the
10  broad range $\phi = -90°$ to $-40°$, $\psi = 120°$ to $180°$ or
$\phi = -90°$ to $-40°$, $\psi = -50°$ to $10°$.  2) Residues were
highly solvent exposed, since mutation of residues in
the core of the enzyme were thought to be more likely
to decrease the enzyme's catalytic efficiency.  3)
15  Residues didn't participate in hydrogen bonding with
other amino acids.  Additionally, based on sequence
alignments with GA's from other organisms [Coutinho and
Reilley, 1994b] only residues which met the above
structural criteria and were not well conserved were
20  selected for mutation.  Ser30 could be aligned with
proline in GAs from *Hormoconis grisea* var *thermoidea*
and *H. resiae GamP* [Coutinho and Reilly, 1994b], which
made it particularly attractive for proline
substitution.
25

**RESULTS**

*Specific activity*

    None of the proline substitution mutations
significantly altered enzyme specific activities of
30  wild-type and mutant GA's at 50°C and pH 4.5.  This
suggests that these mutations did not significantly
alter the enzyme's structure around the active site or
alter its interaction with substrate.

*Irreversible thermal stability*

35      Wild-type and mutant GA's were subjected to
thermal inactivation at pH 4.5 as described in the

experimental protocols. Semilogarithmic plotting of
the percent residual activity against inactivation time
yielded inactivation rate coefficients (kd). Figure 1
shows the relationship between temperature and kd for
5    wild-type and mutant GA's. Based on these data,
activation energies for thermal inactivation (ΔG') were
calculated using transition state theory and melting
temperatures (Tm), the temperature at which the enzyme
was 50% inactivated after 10 minutes were computed
10   (Table 1). As can be seen, the Glu408→Pro mutation
greatly decreased, the Asp345→Pro mutation did not
significantly alter and the Ser30→Pro mutation
increased GA stability.

It should be noted that although Table 1 shows
15   that the Asp345→Pro mutant GA demonstrated slightly
increased ΔG' and Tm, these changes are generally not
significant or that the Asp345→Pro mutant GA is more
stable than wild-type since the kds for this mutant
enzyme at two well separated temperatures (65° and
20   75°C) are essentially indistinguishable from wild-type
(Figure 1).

The proline substitution mutations had different
thermostabilities when measured by their resistance to
irreversible thermal inactivation. When compared to
25   wild-type GA, Glu408→Pro decreased, Asp345→Pro did not
significantly alter and Ser30→Pro increased GA
stability (Figure 1 and Table 1).

Glu408→Pro destabilized GA. As was first
suggested by Schimmel and Flory [1968] and has been
30   expanded by others [MacArthur and Thornton, 1991;
Hurley et al, 1992] proline not only restricts the $\phi,\psi$
values for the site at which it exists, but also the
$\phi,\psi$ values of the preceding residue. These reports
suggest that the ($\phi,\psi$) values for the residue preceding
35   proline should be restricted to approximately $\phi$ = -180°
to -55° and $\psi$= 55° to 180° or $\phi$ = -180° to -55° and

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$\psi$= -30° to -70° for all residues in Xaa-Pro except for
Xaa-Gly, for which the preceding still applies, but is
extended to include $\phi$=45° to 180°. In the published A.
*awamori* var. *X*100 catalytic domain structure [Aleshin
5    et al., 1992], Asp408 ($\phi$=-65°, $\psi$=146°) which aligns
with Glu408 in A. *awamori* GA, has $\phi,\psi$ values within
ranges acceptable for proline. However, the preceding
residue Gly407 ($\phi$=80°, $\psi$=-5°) has $\phi,\psi$ outside
acceptable ranges for positions preceding proline. It
10   is not surprising then, that the Glu408→Pro
destabilized GA. Additionally, X-ray crystallography
suggests that position 408, in the closely related A.
*awamori* var. *X*100 GA[2], lies within a $\beta$-strand; a site
not well suited for proline substitution.
15        Asp345 ($\phi$ = -65°, $\psi$ = -26°) and the preceding
Thr344 ($\phi$ = -116°, $\psi$ =178°) have $\phi,\psi$ angle values lay
well within allowed values for proline substitution at
position 345. However, the Asp345→Pro mutant GA did
not demonstrate stability significantly different from
20   wild-type GA. This is particularly unexpected since
position 345 lies at the N-terminus of an $\alpha$-helix[2]; a
position previously shown to be particularly favorable
for proline substitution [Watanabe et al, 1994].
          Ser30 ($\phi$ = -49°, $\psi$= 130°) is preceded by Val29 ($\phi$
25   = -127°, $\psi$ = 46°) both of which have acceptable $\phi,\psi$
angle values except Val29 $\psi$ = 46° which is slightly
smaller than ideal for proline substitution at position
30.
          In summary, when expressed in *Saccharomyces*
30   *cerevisiae*, Glu408→Pro greatly decreased, Asp345→Pro,
did not significantly alter and Ser30→Pro strongly
stabilized the enzyme. The Ser30→Pro mutant GA showed
a significantly decreased rate of irreversible thermal
inactivation when analyzed between 65° and 77.5°C
35   without decreased enzyme activity. At 65°C a 1.7-fold
decrease in thermal inactivation rate coefficients was

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seen and the activation energy for thermal inactivation
was increased by 1.6 kJ/mol relative to wild-type GA.

## EXAMPLE 2
### ENGINEERED DISULFIDE BONDS

5          The following example is an exemplar of the
methods and procedures that are used in the analysis of
an individual mutation of a glucoamylase.  The process
of GA thermoinactivation is thought to be dominated by
10    formation of enzymes with incorrect conformation [Munch
and Tritsch, 1990].  Previous work supported this
hypothesis.  Site-directed mutagenesis has been used to
eliminate sites of deamidation and peptide hydrolysis
Chen et al., 1994 a,b).  The corresponding mutations
15    Asn182→Ala and Asp257→Glu had reduced irreversible
thermoinactivation rates at pH 4.5 below 70°C but
increased rates above 70°C. Thus GA thermoinactivation
is predominantly caused by "scrambled" structures
rather than by deamidation and peptide hydrolysis.
20    Furthermore, mutations Gly137→Ala, Gly139→Ala and
Gly137/139→Ala/Ala, made to reduce helix flexibility,
showed increased thermostability up to 75°C (Chen et
al., 1996) apparently by slowing down the formation of
incorrect structures.
25          To improve protein thermostability by preventing
formation of incorrect structures, several strategies
have been proposed including introducing covalent
linkage such as disulfide bonds (Perry and Wetzel,
1984; Wetzel, 1987; Matsumura et al., 1989, Clarke and
30    Fersht, 1993).
There are a total of nine cysteine residues in A.
awamori GA, eight of which form disulfide-linked pairs,
which are assumed to enhance the folding and stability
of GA, residues 210 and 213, 262 and 270, 222 and 449
35    [Aleshin et al., 1992] and 509 and 604 [Williamson et
al., 1992b].  In this Example, additional disulfide

bonds are introduced into GA to explore the effect on
thermostability and catalytic activity.  Two engineered
disulfide bond mutants designated A27C/N20C
(abbreviated S-S) and A471C/T72C were constructed.  The
5    new disulfide bond formed by A27C/N20C connects the C-
terminus of helix 1 (Asn20) and a turn where residue
Ala27 is located, while A471C/T72C bridges the N-
terminus of helix 3 and the end of the 30-residue
highly O-glycosylated belt region together.  The
10   disulfide bonds are formed spontaneously after
fermentation and have different effects on GA
thermostability and catalytic activity.

**Site-Directed Mutagenesis**:  Site-directed mutagenesis
was performed as described herein above.
15   Oligonucleotide primers used are: 5'-CGT ACT GCC ATC
CTG TGT AAC ATC GGG GCG GA-3' (N20C, AAT→TGT) (SEQ ID
No:6), 5'-ATC GGG GCG GAC GGT TGT TGG GTG TCG GGC GCG-
3' (A27C, GCT→TGT) (SEQ ID No:7), 5'-CGA AAT GGA GAT
TGC AGT CTC-3' (T72C, ACC→TGC) (SEQ ID No:8), 5'-G AGT
20   ATC GTG TGT ACT GGC GGC ACC-3' (A471C, GCT→TGT) (SEQ ID
No:9), with the underlined letters indicating the
nucleotide mutations.

**SDS-PAGE and Thio-titration**:  SDS-PAGE was carried out
using 0.75 mm thick 10% polyacrylamide gels following
25   the method of Garfin [1990].  For thio-titration, GA at
2 mg/ml concentration was denatured by boiling in
denaturing solution containing 2% SDS, 0.08 M sodium
phosphate (pH 8.0) and 0.5 mg/ml EDTA [Habeeb, 1972]
with or without 50 mM DTT [Pollitt and Zalkin, 1983]
30   for 10 min.  The denatured GA (reduced or non-reduced)
was concentrated using Centricon 30 concentrators
(Amicon, MA, USA) and the reduced GA was applied to
Bio-spin 30 chromatography columns (Bio-Rad, CA, USA)
pre-equilibrated with denaturing solution to remove
35   DTT.  The resulting solution as well as the non-reduced
denatured GA sample were divided into two portions.

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One portion was used for a protein concentration assay
and the other portion was assayed for thio reduction by
mixing with 4 mg/ml DTNB in denaturing solution with a
30:1 volume ratio, followed by incubation at room

5    temperature for 15 minutes, and absorbance measurement
at 412 nm with a molar absorptive value of 13,600 $M^{-1}cm^{-1}$
[Habeeb, 1972].

**GA Activity Assay**: As described herein above,
maltose was used as substrate in enzyme kinetics

10   studies, with concentrations ranging from 0.2 $K_m$ to 4 $K_m$
at 35°C and pH 4.5 as described previously [Chen et al.,
1994b]. Kinetics parameters were analyzed by the
program ENZFITTER. In residual enzyme activity assays,
the conditions are the same as in the enzyme kinetics

15   studies except that only one concentration of maltose
(4%) is used as substrate. Specific activity assays
were carried out with 4% maltose as substrate at 50°C
and pH 4.5. One unit (IU) was defined as the amount of
enzyme required to produce 1$\mu$mol glucose per min under

20   the conditions of the assay. To compare the
temperature optima of catalytic activities of wild-type
and mutant GA, activities were assayed at pH 4.5 with
4% maltose as substrate at different temperatures.

**Irreversible Thermoinactivation**: As described

25   herein above, purified wild-type or mutant GA proteins
were incubated at five different temperatures from 65°C
to 75°C at 2.5°C intervals at 40 $\mu$g/ml in 0.05 M NaOAC
buffer (pH 4.5). At six different time points,
aliquots of the incubating enzyme were removed, quickly

30   chilled on ice, stored at 4°C for 24 hours, and
subjected to residual activity assay. The irreversible
thermoinactivation of GA obeyed first-order kinetics
[Chen et al., 1994b]. Thermoinactivation rate
coefficients, $k_d$ were determined as described previously

35   [Chen et al., 1994b].

**Computer Modeling and Three-dimensional View of**

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**Mutated Residues**: The candidate residues of *A.
awamori* GA to form disulfide bond were modeled with the
crystal structure of *A. awamori* var. *X100* GA [Aleshin
*et al.*, 1992] (*1gly* in the Brookhaven Protein Data

5   Bank) as reference by the SSBOND program (Hazes and
Dijkastra, 1988) installed in a DEC 3100 workstation.

  **Selection of Mutation Site**: Residues Asn20, Ala27
and Thr72, Ala471 were chosen to be replaced with
cysteine. After the analysis of crystal structure of

10  *A. awamori* var. X100 GA [Aleshin *et al.*, 1992] by the
program SSBOND, 132 pairs of residues were found that
could potentially be sites for a disulfide bond. Pairs
containing glycine were discarded on the assumption
that glycine may be required for flexibility at that

15  site. Also, the residues involved in hydrogen bonds
and electrostatic interactions were eliminated.
Residues 20 paired with 27 as well as 72 paired with
471 were chosen as candidates for disulfide bond
formation according to the geometrical analysis. Amino

20  acid sequence alignment among related GAs showed that
there is a disulfide bond between position 20 and 27 in
*Neurospora crassa* [Coutinho and Reilly, 1994b], which
suggested that introducing disulfide bond between
position 20 and 27 would not cause unfavored

25  interactions there in *A. awamori* GA. Furthermore, the
20/27 disulfide bond would link the C-terminus of helix
1 and the conserved S1 fragment of GA involved in
substrate binding [Coutinho and Reilly, 1994a] to form
a loop, near another loop very critical for catalysis

30  containing Trp 120, a residue involved in substrate
binding [Sierks *et al.*, 1989]. Therefore, the proposed
20/27 disulfide bond was expected to stabilize GA by
keeping the correct conformation for catalysis and
substrate binding.

35    Another further candidate for a disulfide bond
pair was between positions 471 and 72. This disulfide

bond would link the N-terminus of helix 3 and the end
of the 30-residue (440-470) highly O-glycosylated belt
region to form a loop.  This disulfide bond also would
make an additional linkage between the catalytic domain
5    and the O-glycosylated linker.  This O-glycosylated
linker has been proved to be important for GA
thermostability by limiting the conformational space
available to the GA unfolded peptide [Semimaru et al.,
1995 and Williamson et al., 1992].  This disulfide bond
10   could have a globe effect on the thermostability of GA
because of this linkage.  The side chain -OH group of
Thr72 in A. awamori var. X100 GA [Aleshin et al., 1992]
is hydrogen bonded to the main chain N atom of Asp73.
In A. awamori GA, however serine is found at residue 73
15   in place of Asp.  It is possible that the hydrogen bond
between residues 72 and 73 does not exist in A. awamori
GA, and therefore replacing Thr72 with Cys will not
disturb this interaction.  This hydrogen bond is
apparently not critical for GA since Thr72 is replaced
20   by Ala, Lys or Val in other GAs [Coutinho and Reilly,
1994b].

**Engineered Disulfide Bonds Were Formed
Spontaneously**: After GA purification, the engineered
disulfide bonds were found to be formed spontaneously
25   by the following two approaches.

First, the mutant A471C/T72C has faster mobility
than wild-type during SDS-PAGE under non-reducing
conditions, suggesting that an additional disulfide
bond forms a new loop retarding the migration.  The
30   possibility that a truncated enzyme was formed in this
case was eliminated by DNA sequencing of the mutant
cDNA and MALDI analysis.  The MALDI data showed that
the mutant GA had the same molecular weight as wild-
type GA.  Mutant A27C/N20C has the same migration as
35   wild-type GA, which may be because the additional loop
caused by the engineered disulfide bond is too small

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(seven residues) to affect migration.

Second, the new disulfide bonds were demonstrated
by thio group titration. Comparing the numbers of free
thio groups before and after the treatment of reducing
reagent DTT, the total disulfide bonds in mutant and
wild-type GA were deduced as reported in Table 2.
Wild-type, A27C/N20C, and A417C/T72C GA have in total
8.6, 10.9, and 10.4 free thio groups respectively
according to the [SH]/molecule ratio in the presence of
reducing reagent DTT (Table 2). In the absence of DTT
the numbers are 0.9, 0.9 and 1.3, respectively (Table
2). This suggested that the number of disulfide bonds
among wild-type, A27C/N20C and A471C/T72C are 4, 5 and
5, respectively. Therefore, the introduced cysteine
residues formed disulfide bonds instead of remaining
free thiols.

**Enzymatic Activity and Optima Temperature of
Catalysis**: The enzymatic properties of double
mutations A27C/N20C and A471C/T72C were not changed
compared with wild-type at 35°C and 50°C as shown in
Table 3, while single mutations had significant reduced
activity. Mutant A27C/N20C and A471C/T72C had specific
activities at 50°C and kinetic parameters at 35°C very
close to wild-type GA (Table 3). The single mutant
A27C had slightly increased $K_m$ but the same $k_{catt}$ value
as wild-type GA, and thus a reduced $k_{cat}/K_m$ ratio of
~30%. Mutant N20C had the same $K_m$ but both a decreased
$k_{cat}$ and $k_{cat}/K_m$ ratio and a decreased specific activity
at 50°C of more than 50%.

**Irreversible Thermoinactivation of GA**: The
irreversible thermoinactivation of wild-type and mutant
GA was studied at 65°C, 67.5°C, 70°C, 72.5°C and 77.5°C
with first-order irreversible theremoinactivation
coefficients $k_d$ shown in Figure 2. Mutants A27C,
A27C/N20C and A471C/T72C had smaller $k_d$ values than did
wild-type GA within the measured temperature range,

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which means the activity decayed more slowly than wild
type, while mutant N20C had greater $k_d$ value than wild-
type at all temperatures except 75°C, which means that
N20C decayed faster than wild-type.

5          Table 4 shows the activation enthalpy ($\Delta H_\ddagger$),
entropy ($\Delta S_\ddagger$) and free energy of unfolding ($\Delta G_\ddagger$) at 65°C
and 75°C of wild-type and mutant GAs, calculated
according to transition-state theory.  The enthalpies
of N20C and A27C/N20C decreased by 42 and 24 KJ/mol

10     respectively, while no significant change occurs for
A27C and A471C/T72C.  Mutants N20C and A27C/N20C had
decreased entropy of 115 kJ/mol and 75 kJ/mol
respectively, while entropy of mutants A27C and
A471C/T72C showed no significant change. Mutant A27C

15     and A471C/T72C  had a slightly higher $\Delta G'$ than wild-type
GA at 65°C and 75°C (<0.5 kJ/mol), while the $\Delta G'$ of
A27C/N20C was higher than that of wild-type by 1.5 and
2.2 kJ/mol at 65°C and 75°C respectively.  Mutant N20C
had a decreased $\Delta G'$ by 3.0 and 1.8 kJ/mol at 65°C and

20     75°C, respectively, compared with wild-type GA.
Therefore, the engineered disulfide bond mutant
A27C/N20C significantly increased GA thermostability
compared with wild-type GA while the single mutants
produced either a slight increase (A27C) or a slight

25     decrease (N20C) in thermostability.  The other
disulfide bond mutant had the thermostability identical
to wild-type GA.

## EXAMPLE 3

### MUTATION A27C/N20C IN COMBINATION WITH OTHER MUTATIONS

30

In previous studies applicants have constructed
the thermostable mutants G137A [Chen et al., 1996] and
S436P (Li et al., 1996), which have the potential to be
combined and improve thermostability additively.  In

35     this Example, these mutations are combined with each
other and with A27C/N20C (S-S; Example 2) to test their

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effects (cumulative/additive) on thermostability and GA
activity.

Enzymatic Activity and Optima Temperature of
Catalysis: The combined mutants A27C/N20C/G137A and
5    A27C/N20C/S436P had increased specific activity while
mutant G137A/S436P had similar specific activity to
wild-type GA (Table 3). The double mutants A27C/N20C
and A471C/T72C as well as the combined mutant
A27C/N20C/G137 had changed optimal temperatures for
10   catalysis.

Relative activity assays at temperatures from 60°C
to 74°C (Figure 3) showed that wild-type, mutant
A27C/N20C and A471C/T72C had the highest activity at
71°C, 72°C and 72.5°C, respectively. From 60°C to
15   67.5°C, mutant and wild-type GA had very similar
activities. However, when the temperature was above
70°C, their relative activities differed substantially.
Mutants A27C/N20C and A27C/N20C/G137A had higher
activity than wild-type consistently from 70°C to 76°C
20   with a peak at 72.5°C, while mutant A471C/T72C had
activity lower than wild-type from 70°C to 71°C and 73°C
to 74°C but higher at 72°C which is its optimal
temperature. Thus mutant GAs A27C/N20C, A471C/T72C and
the combined mutant A27C/N20C/G137A had increased
25   temperature optima above wild-type GA by 1.5°C.

Irreversible Thermoinactivation of GA: The
irreversible thermoinactivation of wild-type and mutant
GA was studied at 65°C, 67.5°C, 70°C, 72.5°C and 77.5°C
with first-order irreversible theremoinactivation
30   coefficients $k_d$ shown in Figure 2. Mutants A27C,
A27C/N20C and A471C/T72C, A27C/N20C/G137A,
A27C/N20C/S436P and G137A/S436P had smaller $k_d$ values
than did wild-type GA within the measured temperature
range, which means the activity decayed more slowly
35   than wild type, while mutant N20C had greater $k_d$ value
than wild-type at all temperatures except 75°C, which

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means that N20C decayed faster than wild-type.

Table 4 shows the activation enthalpy ($\Delta H_t$),
entropy ($\Delta S_t$) and free energy of unfolding ($\Delta G_t$) at 65°C
and 75°C of wild-type and mutant GAs, calculated
5    according to transition-state theory.

The helix flexibility mutant G137A showed additive
thermostability when combined with either S436P or
A27C/N20C. The combination S436P with A27C/N20C did
not show additivity.

10

## EXAMPLE 4
### FURTHER STUDIES WITH COMBINED MUTATIONS

To further investigate whether individual
stabilizing mutations can cumulatively stabilize
15   *Aspergillus awamori* glucoamylase (GA), mutant enzymes
were constructed containing combinations of
thermostabilizing mutations. Previous work has shown
that the following mutations stabilize GA as
demonstrated by decreased irreversible thermal
20   inactivation rates when inactivated in the absence of
carbohydrate: Ser30→Pro (S30P; Example 1), Gly137→Ala
(G137A), and Asn20→Cys/Ala27→Cys (which creates a
disulfide bond between residues 20 and 27 and is
therefore noted as S-S for convenience; Example 2). To
25   investigate whether individual stabilizing mutations
can cumulatively stabilize GA, additional combined
mutant enzymes were prepared utilizing the these three
mutations.

**Site-directed mutagenesis:** The S-S/S30P/G137A combined
30   mutant was constructed using the S-S/S30P
oligonucleotide listed above and a single stranded DNA
template derived from a pBluescript II KS(+) vector
with a 1.7 kb XhoI→BamHI DNA fragment coding for the GA
catalytic domain which already contained mutations
35   conferring the S30P and G137A amino acid substitutions.
The presence of the individual mutations was confirmed

by sequencing and each mutated GA gene fragment was
subcloned into YEpPM18 [Cole et al., 1988] and
transformed into *S. cerevisiae*.

**Thiol analysis:**  10 nmol of wild-type, S-S/S30P and S-

5   S/S30P/G137A mutant GAs were incubated in 0.2 mM 5,5'-
dithiobis(2-nitrobenzoic acid), 6 M GdnHCl, and 50mM
Tris, pH 8 in duplicate [Fierobe et al., 1996].  The
thiol concentration was calculated from a standard
curve established using 0-30$\mu$M cysteine.

10  **Irreversible thermal inactivation:**  Wild-type and
mutant GAs were subjected to thermal inactivation at
six or seven temperatures between 65° and 80°C at 2.5°C
intervals in duplicate.  Following 24 hours at 4°C, the
residual activities of the inactivated samples were

15  analyzed at 35°C along with a corresponding sample
which had not been inactivated [Chen et al, 1996].

**Saccharification analysis:**  Saccharifications were
performed in duplicate using stirring heating blocks
and tightly sealed vials to prevent evaporation.  Eight

20  $\mu$g/ml of wild-type and mutant GAs were assayed using
28% (w/v) Maltrin DE 10 maltodextrin in 0.05 M NaOAc pH
4.5 as substrate.  At various times, sample was
removed, diluted appropriately in 0.05 M NaOAc pH 4.5
and the reaction stopped by adding 100$\mu$l of diluted

25  sample to 40 $\mu$l of 4.0 M Tris-Cl, pH 7.0.  The glucose
concentration was determined by a glucose oxidase/*o*
dianisidine assay [Banks and Greenwood, 1971].

**RESULTS**

*Enzyme activities*

30      Table 5 shows the specific activities of the wild-
type and mutant GAs at 50°C and pH 4.5 using maltose as
substrate.  None of the mutant GAs demonstrated reduced
enzyme activity and the S30P/G137A and S-S/S30P/G137A
mutants were somewhat more active than wild-type at

35  50°C.  To further investigate this observation, the
activities of these mutant enzymes were assayed at

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various temperatures between 35° and 68°C (Figure 4).
The S30P/G137A and S-S/S30P/G137A mutant GAs were more
active than wild-type at all temperatures examined.
*Thiol analysis*

5       The formation of a disulfide bond between
positions 20 and 27 in the Asn20→Cys/Ala27→Cys mutant
GA has been confirmed (Example 2). Table 6 shows the
results of thiol analysis for the combined mutants S-
S/S30P and S-S/S30P/G137A. A. *awamori* GA has one free
10      cysteine at position 320. The combined mutant GAs show
slightly higher thiol content per molecule than wild-
type which may reflect less than complete disulfide
bond formation between positions 20 and 27. However,
if the disulfide bond was completely unformed, the
15      [SH]/protein would be expected to rise to approximately
three with the addition of two free cysteine residues.
Therefore, we conclude that the disulfide bridge is
formed at 70-80% the expected theoretical yield for
complete formation.
20      *Irreversible thermal inactivation*

        Wild-type and mutant GAs were subjected to thermal
inactivation at pH 4.5 between 65° and 80°C.
Semilogarithmic plotting of residual activity versus
inactivation time yielded inactivation rate
25.     coefficients (*kd*). Figure 5 shows the effect of
temperature on *kd* for wild-type and mutant GAs. As can
be seen, the combined mutants are significantly more
stable than the individual mutant enzymes.
Additionally, the temperature at which the enzymes were
30      50% inactivated after 10 minutes (Tm) was calculated by
extrapolation from the thermal inactivation plots and
transition state theory was used to calculate
activation energies for thermal inactivation (ΔG').
Table 7 shows the changes in ΔG' (ΔΔG') and Tm for the
35      combined mutant GAs relative to wild-type GA. These
data clearly demonstrate that combining the individual

stabilizing mutations can cumulatively stabilize the
enzyme.

*Saccharification analysis*

Figure 6 shows the results of saccharification
analysis at 55° and 65°C for wild-type, S30P/G137A and
S-S/S30P/G137A GAs using the industrial DE 10
maltodextrin substrate Maltrin M100 (28% w/v) from
Grain Processing Corporation.  Complete conversion of
28% w/v DE 10 maltodextrin to glucose would result in a
1.71 M glucose syrup however, previous saccharification
analyses in our laboratory have demonstrated that wild-
type GA results in approximately 90% theoretical
maximum glucose yield at 55°C (not shown).  At 55°C no
significant difference in glucose production was
observed between the wild-type and mutant enzymes.
However, at 65°C the mutant GAs produced 8-10% more
glucose than wild-type although none of the enzymes
tested produced as much glucose as at 55°C probably due
to thermal inactivation at the elevated reaction
temperature.

In summary these data show that the S30P/G137A
double mutant enzyme was more stable than either single
mutant GA when analyzed for resistance to irreversible
thermal inactivation between 65°C and 80°C.  The  S-
S/S30P combined GA mutant was also more stable than
either the S30P or the S-S mutant GAs.  The S-
S/S30P/G137A combined mutant was the most stable GA
variant constructed, particularly at temperatures above
70°C when inactivated in a buffer system lacking mono-
or polysaccharides.  Saccharification analysis showed
that the mutant enzymes performed better at elevated
temperatures than wild-type GA.  Importantly, none of
the combined mutant GAs showed decreased enzyme
activity when analyzed at 50°C.

**Discussion**

*Sites of mutation*

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As described in Example 2, the mutations Asn20→Cys
and Ala27→Cys form a disulfide bond between the C-
terminus of α-helix one and an extended loop between α-
helices one and two.  S30P and G137A were designed to
5   stabilize the enzyme by reducing its conformational
entropy of unfolding and are the most stabilizing in a
series of proline substitution (Xaa→Pro) and Gly→Ala
mutations respectively.  Ser30 is located at the second
position of a type II β-turn on an extended loop
10   between α-helices one and two and Gly137 is located in
the middle of the fourth α-helix.

It is of particular importance to note the
positions of the S30P and the disulfide bond forming
mutations.  The disulfide bond is formed between
15   positions 20 and 27; relatively close to position 30.
The fact that both the disulfide bond forming mutations
and the S30P stabilize GA suggests that this region of
the enzyme is critical for irreversible thermal
inactivation and may represent a region of local
20   unfolding important for thermal inactivation.
Additionally, previous investigators have suggested
that a disulfide bond should not be engineered within
four amino acids of a proline in primary sequence
[Balaji et al, 1989].  This Example demonstrates that
25   this rule is not absolute since thiol analysis showed
that the disulfide bond was formed in the S-S/S30P and
S-S/S30P/G137A combined mutants and thermal
inactivation studies showed the stabilizing effects of
the mutations were cumulative.
30   *Cumulative stabilization*

Previous work by Applicants has shown that
combining two stabilizing mutations does not
necessarily stabilize GA [Chen et al, 1996].  The
present study, however, demonstrates that combining
35   stabilizing mutations, even mutations very close to
each other in the protein, can cumulatively stabilize

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GA as measured by resistance to irreversible thermal inactivation.

The S30P/G137A mutant showed more than additive stabilization at low temperatures (65°-70°C), but less than additive stabilization at high temperatures (77.5°-80°C) (Figure 5A and Table 7). At 80°C the inactivation rate for the S30P/G137A combined mutant was nearly identical to the S30P individual mutant protein. This indicates that both regions are very important for low temperature thermal inactivation, but at high temperatures inactivation became governed by other processes.

It was somewhat surprising that combining the S30P with the disulfide bond forming mutations resulted in cumulative stabilization. This is not only because the engineered disulfide bond is so close to the engineered proline as is discussed above, but also because both are targeting the same region of the protein (ie: the extended loop between α-helices one and two). It was expected that either the disulfide bond or S30P stabilized this region maximally, and further stabilization at this site would not result in a functionally more stable enzyme. As can be seen in Figure 5B, this was not the case. Combining the mutations resulted in roughly additive stabilization at all temperatures examined between 65° and 80°C.

The S-S/S30P/G137A combined mutant was no more stable than S30P/G137A GA at low temperatures (65°-70°C), but was slightly more stable at higher temperatures (75°-80°C) (Figure 5C and Table 7). Interestingly, the S-S/S30P GA is also more stable than S30P/G137A GA at high temperatures. Therefore, it appears that the introduced disulfide bond is particularly effective at stabilizing GA at high temperatures.

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## EXAMPLE 5

### INDUSTRIAL APPLICATION

To determine whether the thermal stabilizing
mutations: S30P/G137A and S-S/S30P/G137A would enhance
5       GA performance under industrial conditions wild-type
and mutant enzymes were subjected to high temperature
saccharifications (Figure 6). Saccharification
analysis showed that the mutant enzymes out-performed
wild-type at 65°C but not at 55°C likely due to their
10      increased stability.

### Conclusion

The S30P/G137A double mutant cumulatively
stabilized GA as demonstrated by decreased irreversible
thermal inactivation rates relative to either
15      individual mutant enzyme when analyzed between 65°C and
80°C. Similarly, the S-S/S30P combined mutant also
demonstrated cumulative stabilization. The S-
S/S30P/G137A combined mutant was more stable than
either of the "double" mutants, particularly at
20      temperatures above 70°C. The S-S/S30P combined mutant
had the same activity as wild-type and the S30P/G137A
and S-S/S30P/G137A mutants increased enzyme activity by
10-20% when assayed between 35° and 68°C. The
S30P/G137A and S-S/S30P/G137A mutant GAs decreased
25      thermal inactivation rates approximately three fold
relative to wild-type when inactivated in the presence
of 1.71M glucose at 65°C. Additionally, at 55°C no
difference in glucose yield was observed between these
mutant GAs and wild-type for the saccharification of
30      the industrial substrate Maltrin M100, whereas at 65°C
the S30P/G137A and S-S/S30P/G137A GAs produced 8-10%
more glucose than wild-type.

## EXAMPLE 6

35      ### MUTATIONS WITH INCREASED SELECTIVITY

Interactions between substrates and charged

residues at subsites 1 and 2 of GA play a very
important role in substrate specificity since the
catalytic site is located between these sites.
Therefore mutations were designed and analyzed to

5      determine residues within these regions where mutations
would increase selectivity of the enzyme reaction. In
addition, several mutations that were designed to have
thermostability were also screened for selectivity, as
well as mutations designed to increase the pH optimum.

10     *Site-directed mutagenesis*: Site-directed
mutagenesis was performed as described herein above.
The following mutagenic oligonucleotide primers were
synthesized at the Iowa State University Nucleic Acid
Facility: 5'-GGT CTC GGT GAG CCC A$\underline{G}$G TTC AAT GTC GAT-3'

15     (Lys108→Arg; SEQ ID No:10), 5'-GGT CTC GGT GAG CCC A$\underline{T}$G
TTC AAT GTC GAT-3' (Lys108→Met; SEQ ID No:11), 5'-GAG
GAC ACG TAC T$\underline{GG}$ AAC GGC AAC CCG-3' (Tyr312→Trp; SEQ ID
No:12), and 5'-TAC CCT GAG GAC ACG TAC $\underline{AAC\ GGC\ AAC\ GGC}$
$\underline{AAC\ TCG\ CAG}$ GGC AAC CCG TGG TTC CTG TGC-3' (311-314

20     Loop; SEQ ID No:13), the underlined letters indicating
the changed or added nucleotides.

**Results**

*Enzyme kinetics*

As shown in table 11, the kinetic parameters $k_{cat}$

25     and $K_M$ for the hydrolysis of $G_2$ to $G_7$, as well as $iG_2$ in
0.05 M acetate buffer, pH 4.4, at 45°C are given in
Table 8. The 311-314 Loop mutant had $k_{cat}$ values 50-80%
for all α-(1,4)-linked substrates and only 30% for $iG_2$,
$K_M$ values 50-75% for all substrates. The $k_{cat}$ values for

30     Gly137→Ala/Ser30→Pro GA are 10-30% more, generally,
than that of wild-type GA for all substrates. The $K_M$
values of Gly137→Ala/Ser30→Pro GA are about half to
twofold for all the α-(1,4)-linked substrates and
essentially reached the wildtype level for $iG_2$. The $k_{cat}$

35     values for the GA engineered to carry the triple
mutation, S-S/Gly137→Ala/Ser30→Pro, ranged from 80 to

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120% generally for all substrates, and the $K_M$ values are
30-80% for all substrates compared to wild-type GA.
The $k_{cat}$ values for S-S GA are 85-110% for all
substrates, and the S-S GA $K_M$ values are generally 90-

5    110% for all substrates.  However, the S-S GA $K_M$ values
are 140% for $G_5$ and 190% for $G_6$.  Values of $k_{cat}/K_M$ are
75-105%, 60-110%, 60-110%, and 60-120% for the
Tyr312→Trp mutation, the combined Ser30→Pro/Gly137→Ala
double mutation, the combined S-S/Ser30→Pro/Gly137→Ala

10   triple mutation, and the S-S engineered GA,
respectively.  The catalytic efficiencies for the 311-
314 Loop GA are 85-120% for all the $\alpha$-(1,4)-linked
substrates, and only 50% for $iG_2$, compared to wild-type
GA.

15       Table 8 shows the ratios of the catalytic
efficiencies for $G_2$ to $iG_2$ for wild-type and mutant GAs.
GAs engineered with the 311-314Loop mutation and
Lys108→Arg mutation have the highest (240%) and the
lowest (20%) catalytic efficiencies for $\alpha$-(1,4)- over

20   $\alpha$-(1,6)-linked substrates, respectively.  The GAs
engineered with the Tyr312→Trp and S-S mutations show
50% and 20% increases for this ratio, respectively.
All other mutants had lower ratios, indicating poorer
$\alpha$-(1,4)-hydrolytic ability relative to $\alpha$-(1,6)-hy-

25   drolytic ability than wild-type GA.
*Maltooligosaccharide hydrolysis*

         GA engineered with the 311-314Loop mutation or
with the S-S mutation had the highest average glucose
yields (Figure 7).  The 311-314Loop GA had the lowest

30   initial rates for glucose production (64%, 61%, and 82%
compared to wild-type GA at 35, 45, and 55°C,
respectively) due to a specific activity only 60% that
of wild-type GA (data not shown).  Glucose
concentrations decreased after reaching maximal values

35   because of conversion to oligosaccharides.
*Glucose condensation reactions*

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IG$_2$ concentration profiles in 30% (w/v) glucose
condensation reactions at 35, 45, and 55°C were
analyzed.  GAs engineered with the Lys108Arg mutation
had the·highest and the 311-314Loop mutation as well as
5    the S-S mutation the lowest equilibrium iG$_2$
concentrations at all three temperatures.  Tyr312→Trp,
Ser30→Pro/Gly137→Ala, and S-S/Ser30→Pro/Gly137→Ala GAs
exhibited essentially the same equilibrium iG$_2$
concentrations as wild-type GA.  For all the other
10   engineered thermostable GAs tested, Ser436→Pro, S-
S/Ser436→Pro, S-S/Gly137→Ala, and
Gly137→Ala/Ser436→Pro, all reached higher equilibrium
iG$_2$ concentrations than did wild-type GA.  Table 9 shows
the initial rates of iG$_2$ formation in 30% (w/v) glucose
15   condensation reactions.  S-S and 311-314Loop mutant GAs
have the lowest initial rates at all three reaction
temperatures tested.  Lys108→Arg mutant GA showed the
highest initial rates among all the mutant GAs tested
at all three reaction temperatures.  All the tested
20   thermostable GAs except Ser30→Pro/Gly137→Ala and S-
S/Ser30→Pro/Gly137→Ala had much higher initial rates
than wild-type GA at 35°C, but they dropped to slightly
higher or almost the same rate as wild-type GA at 55°C.
*The specificity for a-(1,6)-linkage synthesis over a-*
25   *(1,4)-linkage hydrolysis*

The ratio of the initial rate of iG$_2$ production in
a 30% (w/v) glucose condensation reaction to that of
glucose formation in 30% DE 10 maltodextrin hydrolysis
was calculated to estimate the selectivity for the
30   synthesis of α-(1,6)-linked products over the
hydrolysis of α-(1,6)-linked substrates.  These
iG$_2$/glucose ratios and their relative ratios for wild-
type and mutant GAs are given in Table 9.  K108R and S-
S mutants showed the highest and the lowest relative
35   ratios among wildtype and all the mutant GAs at all
reaction temperatures, respectively.  Therefore, K108R

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had more specificity for α-(1,6)-linkages than α-(1,4)-
linkages and S-S GA had more affinity for α-(1,4)-
linkages than α-(1,6)-linkages.  The 311-314Loop GA
also showed very low relative ratios at these three
5    temperatures.


## EXAMPLE 7
### ADDITIONAL SELECTIVITY MUTATION ANALYSIS
        Utilizing the methods as set forth herein above,
10   additional mutations were screened for selectivity as
shown in Table 10 and Figures 8 and 9.
**Enzyme Kinetics**: The kinetic parameters are seen in
($k_{cat}$ and $K_m$) for the hydrolysis of α-1,6-linked
isomaltose and α-1,4-linked maltooligodextrins (DP2-7)
15   at 45°C and pH 4.4 are given in Table 10.  Mutant Y175F
was active.  The $k_{cat}$ and $K_m$ values were 83-141% and 106-
171%, respectively, that of wildtype for the different
substrates tested and catalytic efficiencies were 69-
102% that of wildtype.  Mutant R241K was also active.
20   Mutant S411G was highly active.  The $k_{cat}$ and $K_m$ values
were 93-129% and 83-203%, respectively, that of
wildtype for the different substrates tested and
catalytic efficiencies were 55-122% that of wildtype.
Mutant S411A had a similar catalytic efficiency ratio
25   as wildtype.  Mutants Y116W, R241K, and S411G had
decreased catalytic efficiency ratios compared to that
of wildtype GA.
**DE 10 Maltodextrin Hydrolysis**: At 55°C, the highest
glucose yield was about 95% reached by engineered GA
30   with mutant S411A at 216 hours compared to the wildtype
yield of about 90% (Fig. 9).  All of the GAs, except
S411A, reached their highest glucose yields rapidly.
The glucose yield of S411A slowly increased for an
extended period of time.  The initial rates of glucose
35   production at 55°C were 5 to 8 times higher that those
at 35°C.

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**Glucose Condensation Reaction**: Glucose condensation
reactions were used to study the ability of wildtype
and mutant GAs to synthesize isomaltose at high glucose
concentrations (Figure 8). The same concentrations of
5    glucoamylases (2.64 $\mu$M) were used as in the hydrolysis
of DE 10 Maltodextrin.

At 55°C, in spite of the different initial rates
of isomaltose production for wildtype, R241K and Y175F,
isomaltose production reached almost the same
10   concentration at the last time point for these three
mutant GAs (Figure 8), indicating that the isomaltose
production was close to equilibrium status. Isomaltose
production for S411A and S411G was much lower than
wildtype and almost linear as it was also at 35°C.
15   Unexpectedly, isomaltose production for Y116W had a
different (lower) equilibrium status compared to
wildtype. The initial rates of isomaltose production
at 55°C were 5 to 7 times greater than those at 35°C.
R241K had a decreased initial rate of isomaltose
20   production at 55°C compared to that of wildtype, and it
also had a lower increase (about 5 times) in the
initial rate of isomaltose production from 35°C to
55°C, compared to the wildtype increase (about 7
times). Y116W, Y175F, S411A and S411G had increased
25   initial rates of isomaltose production or about 7, 6,
and 5 times, respectively from 35°C to 55°C.
**Selectivity**: The ratio of the initial rate of
isomaltose production (from glucose condensation
reactions) to that of glucose production (from
30   hydrolysis of DE 10 maltodextrin) was calculated to
evaluate selectivity for the synthesis of $\alpha$-1,6-linked
products versus the hydrolysis of $\alpha$-1,4 linked
substrates. This ratio represents the ability of a GA
to synthesize isomaltose at a normalized level of DE 10
35   maltodextrin hydrolytic activity.

Mutants Y175F, S411A and S411G had a decreased

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ratio of the initial rate of isomaltose production to
that of glucose production to that of glucose
production by 12%, 35% and 56% at 35°C, respectively,
and a decreased ratio by 24%, 60% and 62% at 55°C,

5      respectively, compared to wildtype. R241K had a very
similar ratio to that of wildtype at both 35°C and
55°C.


## EXAMPLE 8

10         MUTATIONS TO PROVIDE pH OPTIMIZATION

Utilizing the methods as set forth herein above,
additional mutations, S411G, S411A, S411C, S411H, S411D
were screened for increased pH optimum as shown in
Figure 10 and Tables 11 and 12.

15   **Enzyme kinetics**

The kinetic parameters, $k_{cat}$ and $K_m$, for the
hydrolysis of $\alpha$-1,4-linked maltose and maltoheptaose
and $\alpha$-1,6-linked isomaltose at 45°C and pH 4.4 are
given in Table 11. Mutant S411G glucoamylase was

20   highly active compared to wild-type, with an increased
$k_{cat}$ and $K_m$ 13 - 30% and 11 - 59%, respectively, on the
substrates tested. The catalytic efficiencies ($k_{cat}/K_m$)
were 71 - 116% that of wild-type. Mutant S411A
maintained 65 - 74% of wild-type catalytic efficiency

25   with a slightly decreased $k_{cat}$ and a slightly increased
$K_m$. Mutant S411C maintained 54 - 73% of wild-type
catalytic efficiency with a decrease in both the $k_{cat}$
and $K_m$ values. Since mutant S411H and S411D had only
about 6 - 12% of wild-type catalytic efficiency

30   resulting from a seriously decreased $k_{cat}$ and an
increased $K_m$, the kinetic parameters for the hydrolysis
of isomaltose were not determined. Only mutant S411H
and S411D had large increases (5.5 to 7.5 kJ/mol) in
the transition-state energy, $\Delta(\Delta G)$, for the hydrolysis

35   of maltose and maltoheptaose. The large increases of
transition-state energy indicated that the introduction

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of histidine or aspartic acid into position 411
substantially destabilized the binding between GA and
substrate in the transition-state.

**pH dependence of GA activity**

5       The kinetic parameters, $k_{cat}/K_m$ and $k_{cat}$, of the
hydrolysis of maltose by wild-type and mutant
glucoamylases at different pH values were calculated
from initial rates obtained at low (smaller than 0.2 $K_m$)
and high (higher than 10 $K_m$) concentrations of maltose.

10      The effects of pH on the $k_{cat}/K_m$ and $k_{cat}$ of maltose
hydrolysis were used to determine the pK values (Table
12) of both the free enzymes and the enzyme-substrate
complexes. Although wild-type GA had a higher
catalytic efficiency ($k_{cat}/K_m$) than all of the mutant

15      glucoamylases at all of the pH values tested, mutants
S411G and S411A had higher $k_{cat}$ values than that of
wild-type at some pH values. The uncomplexed and
maltose-complexed S411H and S411D showed more narrow
bell-shaped curves than that of wild-type.

20      The effects of pH on the hydrolysis of
maltoheptaose by wild-type, S411G and S411A GAs were
measured to further investigate the change of pK values
and optimum pH of enzyme-substrate complexes using a
long-length substrate. Surprisingly, not only S411G,

25      but also S411A were highly active compared to wild-type
at the optimum pH. Wild-type GA pK1 values (ionization
of the catalytic base) were 2.77, 2.11, and 2.6 for the
free enzyme, the maltose-complexed form, and the
maltoheptaose-complexed form, respectively. The $pK_2$

30      values (ionization of the catalytic acid) of wild-type
were 5.80, 5.85, and 6.78 for the free enzyme, the
maltose-complexed form, and the maltoheptaose-complexed
form, respectively [Bakir et al., 1993, Hiromi et al.,
1966, Sierks and Svensson, 1994]. Compared to wild-

35      type, the S411G mutation increased the pK1 of both the
maltose-complexed form and the maltoheptaose-complexed

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form by approximately 0.6 units, whereas S411G had no
effect on the pK2 of either enzyme-substrate complexes
and only had a minor effect on the pK1 and pK2 of the
free enzyme.  The combined effect of S411G on pK1 and
5    pK2 was an increased optimum pH of both the maltose-
complexed form and the maltoheptaose-complexed form by
approximately 0.3 units.

    The S411G mutation, however, had no effect on the
optimum pH of the free enzyme. S411A and S411C had very
10   similar effects on the pH dependence of maltose
hydrolysis.  S411A and S411C increased the $pK_1$ of the
free enzyme and the maltose-complexed forms by 0.3 -
0.5 and 1.21 units, respectively.  Surprisingly, S411A
and S411C also increased the $pK_2$ of the maltose-
15   complexed form by approximately 0.5 units.  In
addition, S411A increased the $pK_1$ and $pK_2$ of the
maltoheptaose-complexed form by 1.31 and 0.4 units,
respectively.  S411H increased the $pK_1$ of the free
enzyme and maltose-complexed form by 0.33 and 1.47
20   units, respectively; however, it decreased the $pK_2$ of
the free enzyme and the maltose-complexed form by 0.79
and 1.16 units, respectively.  S411D increased the pK1
of the free enzyme and the maltose-complexed form by
0.36 and 1.23 units, respectively.  S411D also
25   decreased the $pK_2$ of the maltose-complexed form by 0.32
units.  For wild-type, S411G, and S411A GAs, the values
of $pK_1$, $pK_2$, and $pH_{opt}$ for the maltoheptaose-complexed
forms were higher than those of the corresponding
maltose-complexed forms by approximately 0.5, 0.9 and
30   0.7 units, respectively.  For S411G and S411A, the
increases in pH optimum (compared to that of wild-type)
obtained using the long-length substrate
(maltoheptaose) were almost the same as that obtained
using the short-length substrate (maltose).
35       All five mutants at position 411 showed a shift of
0.15 to 0.87 units in the optimum pH of the enzyme-

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substrate complex compared to wild-type (Table 12),
mainly due to increased $pK_1$ values. Compared with other
mutants, S411A was the best performing pH mutant.
S411A increased the optimum pH by 0.84 units while also
5    maintaining a high level of both catalytic activity
($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$).

**The hydrolysis of maltodextrin 10**

The hydrolysis of 28% (w/v) maltodextrin was used
to study the pH dependence of GA activity at a high
10   concentration of a long-length substrate. Maltodextrin
10 is a mixture of maltodextrin with an average (and
major) degree of polymerization of 10. The production
of glucose by wild-type and S411A glucoamylases during
the hydrolysis of maltodextrin 10 at 11 different pH
15   values was determined, and used to calculate the
initial rates of glucose production at different pH
values (Figure 10). The production of glucose
increased following a hyperbolic curve. S411A had
higher initial rates of glucose production than wild-
20   type when the pH values were above 6.6 (Figure 10).

Throughout this application, various
publications are referenced by author and year and
patents listed by number. Full citations for the
publications are listed below. The disclosures of
25   these publications and patents in their entireties are
hereby incorporated by reference into this application
in order to more fully describe the state of the art to
which this invention pertains.

The invention has been described in an
30   illustrative manner, and it is to be understood that
the terminology which has been used is intended to be
in the nature of words of description rather than of
limitation.

Obviously, many modifications and variations of
35   the present invention are possible in light of the
above teachings. It is, therefore, to be understood

that within the scope of the appended claims, the
invention may be practiced otherwise than as
specifically described.

TABLE 1

Changes in $\Delta G^{\ddagger}$ and Tm for the mutant GAs relative to wild-type.

| GA form | $\Delta\Delta G^{\ddagger}$ (kJ/mol.) | $\Delta$Tm (°C) |
|---|---|---|
| Ser30→Pro | 1.6 | 1.7 |
| Asp345→Pro | 0.5 | 0.4 |
| Glu408→Pro | -7.2 | -6.7 |

TABLE 2

Summary of DTNB-titratable sulfhydryl groups in wild-type and mutant GA with or without DTT reducing

| Enzymes | [SH]/molecule | | No. of disulfide bonds* |
|---|---|---|---|
| | DTT+ | DTT- | |
| WT | 8.6 | 0.9 | 4 |
| A27C/N20C | 10.9 | 0.9 | 5 |
| A471C/T72C | 10.4 | 1.3 | 5 |

* No. of disulfide bonds= ([SH]/molecule (DTT+)-[SH]/molecule (DTT-))/2

TABLE 3

| Catalytic properties of wild type and mutant GAs. | | | | |
|---|---|---|---|---|
| GA form | Specific Activity (IU/mg GA) | $K_m$ (mM) | $k_{cat}$ (s) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) |
| WT[a] | 20.6±0.2[b] | 0.72±0.03 | 8.67±0.17 | 12.0 |
| A27C | 14.9±1.1 | 0.86±0.11 | 8.02±0.45 | 9.3 |
| N20C | 8.1±0.5 | 0.70±0.05 | 3.97±0.12 | 5.7 |
| A27C/N20C | 18.3±0.7 | 0.90±0.08 | 9.61±0.40 | 10.7 |
| A471C/T72C | 22.7±1.5 | 0.87±0.07 | 10.17±0.40 | 11.6 |
| A27C/N20C /S436 | 22.5±1.8 | N/D[c] | N/D | N/D |
| A27C/N20C /G137A | 24.2±0.8 | N/D | N/D | N/D |
| G137A/S436P | 25.0±0.9 | N/D | N/D | N/D |

[a] Produced in shaking flasks
[b] standard error
[c] not determined

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TABLE 4

| GA form | $\Delta H^{\ddagger}$ (kJ/mol) | $\Delta S^{\ddagger}$ (J/mol·K) | $\Delta G^{\ddagger}$ (65°C) (kJ/mol) | $\Delta G^{\ddagger}$ (75°C) (kJ/mol) |
|---|---|---|---|---|
| WT[a] | 366± 1[b] | 769± 4 | 105.7 | 98.0 |
| A27C | 370±15 | 780±44 | 106.3 | 98.5 |
| N20C | 324±11 | 654±33 | 102.7 | 96.2 |
| A27C/N20C | 342±16 | 694±46 | 107.2 | 100.2 |
| A471C/T72C | 365± 9 | 768±26 | 106.0 | 98.3 |
| A27C/N20C /S436P | 352± 6 | 724±18 | 107.9 | 100.7 |
| A27C/N20C /G137A | 362± 1 | 751± 2 | 108.4 | 100.9 |
| G137A/S436P | 362±20 | 752±57 | 107.7 | 100.2 |
| S436P[c] | 351± 8 | 723±24 | 106.2 | 99.0 |
| G137A[d] | 330± 6 | 661±17 | 106.5 | 99.9 |

Activation parameters for irreversible thermoinactivation of wild-type (WT) and mutant GAs at pH 4.5.

[a] produced by shaking flask
[b] standard error
[c] Li *et al.*, 1996
[d] Chen *et al.*, 1996

Table 5. Specific activities of wild-type and mutant GAs

| GA form | Specific activity[a] (IU/mg) |
|---|---|
| Wild-type | 21.1±0.1 |
| S30P/Gly137A | 24.0±1.2 |
| S-S/S30P | 21.2±0.5 |
| S-S/S30P/G137A | 24.5±0.2 |

[a] Standard deviation resulting from three or more assays

Table 6. Thiol analysis of wild-type and mutant GAs

| GA form | [Protein] (μM) | [SH] (μM)[a] | [SH]/[Protein][a] |
|---|---|---|---|
| Wild-type | 10 | 8 | 0.8 |
| S-S/S30P | 10 | 11 | 1.1 |
| S-S/S30P/G137A | 10 | 13 | 1.3 |

[a] Average of duplicate analysis

Table 7. Changes in free energies for thermal inactivation ($\Delta\Delta G^{\ddagger}$) and temperatures at which the enzyme is 50% inactivated after 10 minutes ($\Delta Tm$) relative to wild-type GA

| GA form | $\Delta\Delta G^{\ddagger}$ [a] (kJ/mol) | $\Delta Tm$ (°C) |
|---|---|---|
| S30P[b] | 1.6 | 1.7 |
| G137A[c] | 0.8 | 1.2 |
| S-S[d] | 1.2 | 1.4 |
| S30P/G137A | 4.5 | 3.5 |
| S-S/S30P | 3.5 | 3.2 |
| S-S/S30P/G137A | 4.4 | 3.9 |

[a] Calculated at 65°C
[b] From Allen et. al.[8]
[c] From Chen et. al.[6]
[d] From Li et. al.[7]

TABLE 8

Kinetic parameters of wild-type and mutant GAs for hydrolysis of maltooligosaccharides DP 2-7 (G2-G7) at 45°C in 0.05 M acetate pH 4.4

| Glucoamylase | $G_2$ | $G_3$ | $G_4$ | $G_5$ | $G_6$ | $G_7$ |
|---|---|---|---|---|---|---|
| **Wild-type** | | | | | | |
| $k_{cat}$ ($s^{-1}$) | 18.6 ± 0.4[a] | 50.8 ± 0.6 | 67.5 ± 1.9 | 61.5 ± 0.33 | 65.9 ± 1.2 | 81.5 ± 1.8 |
| $K_M$ (mM) | 1.09 ± 0.08 | 0.353 ± 0.013 | 0.239 ± 0.017 | 0.094 ± 0.002 | 0.098 ± 0.007 | 0.136 ± 0.009 |
| $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | 17.1 ± 0.9 | 144 ± 4 | 282 ± 13 | 653 ± 10 | 671 ± 36 | 599 ± 27 |
| **Lys108Arg** | | | | | | |
| $k_{cat}$ ($s^{-1}$) | 17.3 ± 0.5 | 32.6 ± 0.9 | 46.6 ± 1.6 | 51.7 ± 1.4 | 55.2 ± 1.4 | 86.2 ± 3.1 |
| $K_M$ (mM) | 1.52 ± 0.11 | 0.570 ± 0.038 | 0.383 ± 0.029 | 0.307 ± 0.019 | 0.276 ± 0.016 | 0.481 ± 0.031 |
| $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | 11.4 ± 0.6 | 57.2 ± 2.5 | 122 ± 5 | 168 ± 6 | 200 ± 8 | 179 ± 6 |
| $\Delta(\Delta G)$[b] (kJ $mol^{-1}$) | 0.92 | 2.10 | 1.91 | 3.08 | 2.75 | 2.74 |
| **Tyr312Trp** | | | | | | |
| $k_{cat}$ ($s^{-1}$) | 17.2 ± 0.3 | 36.8 ± 0.9 | 50.7 ± 0.9 | 50.7 ± 0.8 | 56.0 ± 0.8 | 63.3 ± 0.6 |
| $K_M$ (mM) | 0.940 ± 0.059 | 0.343 ± 0.028 | 0.193 ± 0.010 | 0.100 ± 0.006 | 0.108 ± 0.005 | 0.103 ± 0.003 |
| $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | 18.3 ± 0.90 | 107 ± 6 | 262 ± 9 | 508 ± 22 | 519 ± 20 | 617 ± 1 |
| $\Delta(\Delta G)$ (kJ $mol^{-1}$) | -0.16 | 0.67 | 0.17 | 0.57 | 0.58 | -0.07 |
| **300Loop** | | | | | | |
| $k_{cat}$ ($s^{-1}$) | 14.7 ± 0.3 | 25.9 ± 0.6 | 34.1± 0.8 | 43.0 ± 0.6 | 41.4 ± 0.8 | 41.9 ± 0.7 |
| $K_M$ (mM) | 0.738 ± 0.055 | 0.234 ± 0.019 | 0.114 ± 0.008 | 0.072 ± 0.004 | 0.064 ± 0.005 | 0.083±0.005 |
| $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | 20.0 ± 1.2 | 111 ± 7 | 300 ± 17 | 598 ± 28 | 642 ± 47 | 506 ± 25 |
| $\Delta(\Delta G)$ (kJ $mol^{-1}$) | -0.35 | 0.60 | -0.14 | 0.20 | 0.10 | 0.38 |
| **Ser30Pro/Gly137Ala** | | | | | | |
| $k_{cat}$ ($s^{-1}$) | 25.0 ± 1.1 | 50.2 ± 3.0 | 77.9 ± 2.2 | 77.7 ± 1.6 | 77.0 ± 2.2 | 80.3 ± 2.2 |
| $K_M$ (mM) | 1.62 ± 0.11 | 0.596 ± 0.010 | 0.261 ± 0.020 | 0.175 ± 0.011 | 0.204 ± 0.017 | 0.151 ± 0.013 |
| $k_{cat}/K_M$ ($s^{-1}$ $mM^{-1}$) | 15.5 ± 1.2 | 84.2 ± 3.1 | 299 ± 16 | 444 ± 21 | 377 ± 23 | 533 ± 37 |
| $\Delta(\Delta G)$ (kJ $mol^{-1}$) | 0.27 | 1.42 | -0.15 | 1.02 | 1.52 | 0.31 |

**TABLE 8 (continued)**

**ss/Ser30Pro/Gly137Ala**

| | | | | | | |
|---|---|---|---|---|---|---|
| $k_{cat}$ (s⁻¹) | 23.0 ± 0.9 | 42.1 ± 1.0 | 72.0 ± 2.1 | 72.2 ± 1.0 | 79.5 ± 1.7 | 81.5 ± 1.4 |
| $K_M$ (mM) | 1.66 ± 0.07 | 0.470 ± 0.019 | 0.236 ± 0.007 | 0.172 ± 0.011 | 0.157 ± 0.011 | 0.198 ± 0.010 |
| $k_{cat}/K_M$ (s⁻¹mM⁻¹) | 13.9 ± 0.9 | 89.6 ± 4.2 | 305 ± 17 | 420 ± 13 | 505 ± 26 | 410 ± 15 |
| $\Delta(\Delta G)$ (kJ mol⁻¹) | 0.55 | 1.26 | -0.21 | 1.16 | 0.75 | 1.00 |

**ss**

| | | | | | | |
|---|---|---|---|---|---|---|
| $k_{cat}$ (s⁻¹) | 20.7 ± 0.6 | 40.8 ± 0.9 | 72.1 ± 1.3 | 76.5 ± 0.8 | 76.4 ± 2.1 | 71.8 ± 0.6 |
| $K_M$ (mM) | 1.16 ± 0.10 | 0.394 ± 0.025 | 0.217 ± 0.011 | 0.132 ± 0.005 | 0.184 ± 0.015 | 0.114 ± 0.003 |
| $k_{cat}/K_M$ (s⁻¹mM⁻¹) | 17.8 ± 1.1 | 104 ± 5 | 331 ± 12 | 579 ± 16 | 414 ± 26 | 632 ± 15 |
| $\Delta(\Delta G)$ (kJ mol⁻¹) | -0.10 | 0.88 | -0.42 | 0.32 | 1.28 | -0.14 |

[a] standard error

[b] Change of transition-state energy $\Delta(\Delta G) = -RT\ln[(k_{cat}/K_M)mut/(k_{cat}/K_M)wt]$

TABLE 9

Initial rates of glucose and isomaltose productions in the hydrolysis of 30% (w/v) maltodextrin M100 and 30% (w/v) glucose condensations, respectively, and their relative ratios for wild-type and mutant glucoamylases at 35°C, 45°C, and 55°C.

| Enzymes | Initial rates | | Ratios | |
|---|---|---|---|---|
| | Glucose[a](G1) | Isomaltose[b](iG2) | Ratios | Relative |
| | $(\mu g/mL \cdot h) \times 10^{-3}$ | $(\mu g/mL \cdot h) \times 10^{3}$ | $(iG_2/G1) \times 10^{6}$ | ratios |
| **35°C** | | | | |
| Wild-type | 21.5 ±0.6[c] | 289 ± 5 | 13.5 | 1.00 |
| Lys108Arg | 22.0 ±0.4 | 969 ± 12 | 44.1 | 3.27 |
| Tyr312Trp | 17.9 ±0.5 | 294 ± 4 | 16.4 | 1.21 |
| 311-314Loop | 13.8 ±0.4 | 128 ± 3 | 9.3 | 0.69 |
| Ser30Pro/Gly137Ala | 27.7 ±0.4 | 298 ± 6 | 10.8 | 0.80 |
| S-S/Ser30Pro/Gly137Ala | 30.1 ±0.5 | 245 ± 6 | 8.2 | 0.60 |
| S-S | 31.8 ±0.6 | 135 ± 3 | 4.2 | 0.31 |
| Ser436Pro | 29.9 ±0.6 | 903 ± 12 | 30.2 | 2.23 |
| S-S/Ser436Pro | 31.7 ±0.5 | 824 ± 12 | 26.0 | 1.92 |
| S-S/Gly137Ala | 35.2 ±0.6 | 982 ± 15 | 27.9 | 2.07 |
| Gly137Ala/Ser436Pro | 36.6 ±0.7 | 776 ± 10 | 21.2 | 1.57 |
| **45°C** | | | | |
| Wild-type | 66.2 ±2.2 | 3880 ± 60 | 58.7 | 1.00 |
| Lys108Arg | 50.2 ±2.0 | 6420 ± 110 | 128 | 2.18 |
| Tyr312Trp | 52.6 ±2.1 | 3360 ± 60 | 63.9 | 1.09 |
| 311-314Loop | 40.4 ±1.8 | 1430 ± 40 | 35.3 | 0.60 |
| Ser30Pro/Gly137Ala | 76.3 ±2.7 | 3690 ± 70 | 48.4 | 0.83 |
| S-S/Ser30Pro/Gly137Ala | 84.3 ±3.0 | 3520 ± 60 | 41.7 | 0.71 |
| S-S | 86.3 ±3.3 | 963 ± 28 | 11.2 | 0.19 |
| **55°C** | | | | |
| Wild-type | 156 ±3 | 4890 ± 80 | 31.3 | 1.00 |
| Lys108Arg | 101 ±1 | 8200 ± 120 | 81.0 | 2.59 |
| Tyr312Trp | 110 ±2 | 4440 ± 70 | 40.5 | 1.29 |

TABLE 9 (continued)

| | | | | |
|---|---|---|---|---|
| 311-314Loop | 128 ±2 | 1890 ± 50 | 14.8 | 0.47 |
| Ser30Pro/Gly137Ala | 157 ±3 | 7200 ± 110 | 45.8 | 1.47 |
| S-S/Ser30Pro/Gly137Ala | 167 ±3 | 5690 ± 100 | 34.1 | 1.09 |
| S-S | 164 ±3 | 1230 ± 40 | 7.5 | 0.24 |
| Ser436Pro | 218 ±3 | 4710 ± 80 | 21.6 | 0.69 |
| S-S/Ser436Pro | ND[d] | 5130 ± 100 | ND | ND |
| SS/Gly137Ala | 225 ±3 | 5720 ± 100 | 25.4 | 0.81 |
| Gly137Ala/Ser436Pro | 208 ±3 | ND | ND | ND |

[a]Samples were taken from 30% (w/v) M100 hydrolysis reactions in 0.05 M NaOAc

buffer, pH 4.4; glucose concentrations were determined by glucose oxidase

method.

[b]Samples were taken from 30% (w/v) glucose condensation reactions in 0.05 M

NaOAc buffer, pH 4.4; isomaltose concentrations were determined by HPTLC.

[c]Standard error

[d]Not determined

TABLE 10

Kinetic parameters of wild-type and mutant glucoamylase for hydrolysis of isomaltose and maltooligodextrins of DP 2 - 7

| Enzyme | Isomaltose (iG2) | Maltose (G2) | Maltotriose (G3) | Maltotetraose (G4) | Maltopentaose (G5) | Maltohexaose (G6) | Maltoheptaose (G7) | $k_{cat}/K_m$ (G2) / $k_{cat}/K_m$ (iG2) |
|---|---|---|---|---|---|---|---|---|
| **Wild-type** | | | | | | | | 656 |
| $k_{cat}$ ($S^{-1}$) | 0.72 ±0.01[b] | 20.4 ±0.2 | 48.2 ±0.7 | 64.5 ± 2.9 | 71.8 ± 1.9 | 73.7 ± 2.1 | 72.3 ± 0.9 | |
| $K_m$ (mM) | 23.5 ±0.6 | 1.01 ±0.03 | 0.25 ±0.014 | 0.111± 0.017 | 0.110± 0.010 | 0.107± 0.010 | 0.083 ± 0.004 | |
| $k_{cat}/K_m$ ($S^{-1}$ mM$^{-1}$) | 0.031 ±0.001 | 20.3 ±0.55 | 196 ±9 | 582 ±65 | 654 ±43 | 685 ±47 | 870 ±35 | |
| **Y48F49W** | | | | | | | | ND |
| $k_{cat}$ ($S^{-1}$) | ND[d] | 0.236 ±0.016 | ND | ND | ND | ND | 1.99 ± 0.08 | |
| $K_m$ (mM) | | 9.9 ±1.8 | | | | | 4.9 ± 0.3 | |
| $k_{cat}/K_m$ ($S^{-1}$ mM$^{-1}$) | | 0.024 ±0.003 | | | | | 0.408 ± 0.010 | |
| $\Delta(\Delta G)^c$ (KJ mol$^{-1}$) | | 17.8 | | | | | 20.3 | |
| **Y116W** | | | | | | | | 498 |
| $k_{cat}$ ($S^{-1}$) | 0.69 ±0.02 | 11.7 ±0.2 | 19.4 ±0.3 | 50.9 ± 1.9 | 50.0 ± 1.7 | 53.1 ± 1.9 | 56.0 ± 1.1 | |
| $K_m$ (mM) | 28.8 ±2.5 | 0.98 ±0.06 | 0.20 ±0.01 | 0.20 ± 0.02 | 0.132 ± 0.014 | 0.143 ± 0.017 | 0.118 ± 0.008 | |
| $k_{cat}/K_m$ ($S^{-1}$ mM$^{-1}$) | 0.024 ±0.001 | 12.0 ±0.60 | 98 ±6 | 256 ±17 | 378 ±30 | 372 ±32 | 475 ±25 | |
| $\Delta(\Delta G)$ (KJ mol$^{-1}$) | 0.67 | 1.39 | 1.84 | 2.17 | 1.45 | 1.62 | 1.60 | |
| **Y175F** | | | | | | | | 752 |
| $k_{cat}$ ($S^{-1}$) | 1.02 ±0.05 | 21.2 ±0.2 | 40.0 ±0.6 | 80.1 ± 1.8 | 79.6 ± 1.9 | 76.5 ± 1.5 | 72.1 ± 0.8 | |
| $K_m$ (mM) | 40.1 ±4.3 | 1.13 ±0.04 | 0.29 ±0.02 | 0.187± 0.012 | 0.120± 0.010 | 0.113± 0.008 | 0.095 ± 0.004 | |
| $k_{cat}/K_m$ ($S^{-1}$ mM$^{-1}$) | 0.025 ±0.002 | 18.8 ±0.5 | 136 ±6 | 429 ±19 | 666 ±42 | 677 ±37 | 761 ±27 | |
| $\Delta(\Delta G)$ (KJ mol$^{-1}$) | 0.55 | 0.20 | 0.97 | 0.81 | -0.05 | 0.03 | 0.35 | |

[a] Determined at 45°C in 0.05 M sodium acetate buffer, pH 4.4.

[b] Standard error.

[c] Changes of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.

[d] Not determined.

TABLE 10 (continued)

| Enzyme | Substrate | | | | | | | $k_{cat}/K_m$ (G2) / $k_{cat}/K_m$ (iG2) |
|---|---|---|---|---|---|---|---|---|
| | Isomaltose (iG2) | Maltose (G2) | Maltotriose (G3) | Maltotetraose (G4) | Maltopentaose (G5) | Maltohexaose (G6) | Maltoheptaose (G7) | |
| **R241K** | | | | | | | | |
| $k_{cat}$ (S⁻¹) | 1.34 ± 0.08[b] | 20.1 ± 0.3 | 46.8 ± 1.0 | 71.5 ± 7.2 | 70.7 ± 2.1 | 75.8 ± 2.7 | 80.6 ± 1.6 | 261 |
| $K_m$ (mM) | 39.3 ± 5.8 | 2.27 ± 0.11 | 0.62 ± 0.04 | 0.45 ± 0.09 | 0.19 ± 0.02 | 0.20 ± 0.02 | 0.20 ± 0.01 | |
| $k_{cat}/K_m$ (S⁻¹ mM⁻¹) | 0.034 ± 0.003 | 8.9 ± 0.3 | 76 ± 3 | 164 ± 18 | 368 ± 21 | 373 ± 25 | 411 ± 16 | |
| $\Delta(\Delta G)^c$ (KJ mol⁻¹) | -0.28 | 2.19 | 2.51 | 3.36 | 1.52 | 1.61 | 1.98 | |
| **S411A** | | | | | | | | |
| $k_{cat}$ (S⁻¹) | 0.63 ± 0.02 | 18.9 ± 0.3 | 44.6 ± 0.1 | 58.5 ± 1.6 | 53.1 ± 1.2 | 54.7 ± 1.7 | 59.4 ± 0.6 | 681 |
| $K_m$ (mM) | 27.9 ± 2.9 | 1.26 ± 0.06 | 0.47 ± 0.04 | 0.182 ± 0.014 | 0.120 ± 0.009 | 0.115 ± 0.012 | 0.104 ± 0.004 | |
| $k_{cat}/K_m$ (S⁻¹ mM⁻¹) | 0.022 ± 0.002 | 15.0 ± 0.5 | 94.1 ± 5.4 | 322 ± 18 | 443 ± 27 | 476 ± 41 | 570 ± 17 | |
| $\Delta(\Delta G)$ (KJ mol⁻¹) | 0.84 | 0.80 | 1.94 | 1.56 | 1.15 | 0.96 | 1.12 | |
| **S411G** | | | | | | | | |
| $k_{cat}$ (S⁻¹) | 0.93 ± 0.06 | 23.0 ± 0.4 | 55.1 ± 1.6 | 59.7 ± 1.8 | 75.1 ± 2.1 | 75.9 ± 4.3 | 84.0 ± 2.5 | 402 |
| $K_m$ (mM) | 26.2 ± 2.7 | 1.59 ± 0.08 | 0.50 ± 0.04 | 0.092 ± 0.010 | 0.094 ± 0.010 | 0.125 ± 0.024 | 0.132 ± 0.012 | |
| $k_{cat}/K_m$ (S⁻¹ mM⁻¹) | 0.036 ± 0.004 | 14.5 ± 0.6 | 108 ± 6 | 649 ± 55 | 795 ± 61 | 609 ± 87 | 634 ± 41 | |
| $\Delta(\Delta G)$ (KJ mol⁻¹) | -0.39 | 0.89 | 1.56 | -0.29 | -0.52 | 0.31 | 0.84 | |

[a]Determined at 45°C in 0.05 M sodium acetate buffer, pH 4.4.

[b]Standard error.

[c]Changes of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.

[d]Not determined.

TABLE 11

Kinetic parameters of wild-type and mutant glucoamylases for hydrolysis of isomaltose, maltose and maltoheptaose

| Substrate | Wild-type | Mutant | | | | |
|---|---|---|---|---|---|---|
| | | S411G | S411A | S411C | S411H | S411D |
| **Isomaltose (IG2)** | | | | | | |
| $k_{cat}$ (S$^{-1}$) | 0.72 ± 0.01[b] | 0.93 ± 0.06 | 0.63 ± 0.02 | 0.22 ± 0.01 | ND[d] | ND[d] |
| $K_m$ (mM) | 23.5 ± 0.6 | 26.2 ± 2.7 | 27.9 ± 2.9 | 12.3 ± 0.9 | | |
| $k_{cat}/K_m$ (S$^{-1}$ mM$^{-1}$) | 0.031 ± 0.001 | 0.036 ± 0.004 | 0.022 ± 0.002 | 0.018 ± 0.001 | | |
| Δ(ΔG)[c] (KJ mol$^{-1}$) | - | -0.39 | 0.84 | 1.4 | | |
| **Maltose (G2)** | | | | | | |
| $k_{cat}$ (S$^{-1}$) | 20.4 ± 0.2 | 23.0 ± 0.4 | 18.9 ± 0.3 | 7.78 ± 0.07 | 5.31 ± 0.15 | 4.36 ± 0.05 |
| $K_m$ (mM) | 1.01 ± 0.03 | 1.59 ± 0.08 | 1.26 ± 0.06 | 0.53 ± 0.02 | 3.67 ± 0.25 | 3.58 ± 0.11 |
| $k_{cat}/K_m$ (S$^{-1}$ mM$^{-1}$) | 20.3 ± 0.6 | 14.5 ± 0.6 | 15.0 ± 0.5 | 14.8 ± 0.6 | 1.45 ± 0.06 | 1.22 ± 0.03 |
| Δ(ΔG)[c] (KJ mol$^{-1}$) | - | 0.89 | 0.80 | 0.83 | 6.98 | 7.43 |
| **Maltoheptaose (G7)** | | | | | | |
| $k_{cat}$ (S$^{-1}$) | 72.3 ± 0.9 | 84.0 ± 2.5 | 59.4 ± 0.6 | 33.0 ± 0.5 | 32.4 ± 0.9 | 15.8 ± 0.3 |
| $K_m$ (mM) | 0.083 ± 0.004 | 0.132 ± 0.012 | 0.104 ± 0.004 | 0.070 ± 0.005 | 0.336 ± 0.024 | 0.148 ± 0.009 |
| $k_{cat}/K_m$ (S$^{-1}$ mM$^{-1}$) | 870 ± 35 | 634 ± 41 | 570 ± 17 | 474 ± 25 | 97 ± 5 | 107 ± 5 |
| Δ(ΔG)[c] (KJ mol$^{-1}$) | - | 0.84 | 1.12 | 1.60 | 5.81 | 5.54 |

[a]Determined at 45°C in 0.05 M sodium acetate buffer, pH 4.4.
[b]Standard error.
[c]Changes of transition-state energy $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}]$.
[d]Not determined.

**TABLE 12**

pK values and optimum pH of wild-type and mutant glucoamylases for hydrolysis of maltose and maltoheptaose at 45°C

| | Free enzyme (uncomplexed) | | | Enzyme-substrate complex (maltose-complexed) | | | Enzyme-substrate complex (maltoheptaose-complexed) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $pK_1$ | $pK_2$ | $pH_{opt}$ | $pK_1$ | $pK_2$ | $pH_{opt}$ | $pK_1$ | $pK_2$ | $pH_{opt}$ |
| Wild-type | 2.77 | 5.80 | 4.29 | 2.11 | 5.85 | 3.98 | 2.60 | 6.78 | 4.69 |
| S411G | 3.01 | 5.57 | 4.29 | 2.68 | 5.81 | 4.24 | 3.22 | 6.73 | 4.98 |
| S411A | 3.11 | 5.86 | 4.49 | 3.32 | 6.32 | 4.82 | 3.91 | 7.18 | 5.54 |
| S411C | 3.26 | 5.86 | 4.56 | 3.32 | 6.38 | 4.85 | ND[a] | ND | ND |
| S411H | 3.10 | 5.01 | 4.05 | 3.58 | 4.69 | 4.13 | ND | ND | ND |
| S411D | 3.13 | 5.72 | 4.42 | 3.34 | 5.53 | 4.44 | ND | ND | ND |

[a]Not determined.

TABLE 13

Increases in free energies for thermal inactivation ($\Delta\Delta G^{\ddagger}$) relative
to wild-type GA calculated at 65°C

| GA form | $\Delta\Delta G^{\ddagger a}$ (kJ/mol) |
|---|---|
| S436P | 0.5 |
| S30P | 1.6 |
| G137A | 0.8 |
| S-S | 1.2 |
| S-S/S436P | 2.2 |
| G137A/S436P | 2.0 |
| S30P/G137A | 4.5 |
| S-S/S30P | 3.5 |
| S-S/G137A | 2.7 |
| S-S/S30P/G137A | 4.4 |

[a] $\Delta\Delta G^{\ddagger}$ greater than zero indiactes increased thermostability

TABLE 14

Decrease in the relative ratio of initial rate of isomaltose formation from 30% (w/v) glucose
condensation reactions to that of glucose formation in 30% (w/v) maltodextrin M100 hydrolysis
reactions.

| GA form | Relative ratios[a] |
|---|---|
| Wild-type | 1.00 |
| S-S | 0.24 |
| S30P | 0.77 |
| G137A | 0.54 |
| Y175F | 0.76 |
| 300Loop | 0.47 |
| S411A | 0.40 |
| S411G | 0.38 |
| S436P | 0.70 |
| S-S/G137A | 0.81 |
| G121A/S411G | 0.44 |

All the above reactions were carried out in 0.05M sodium acetate buffer, pH4.4, at 55°C.
[a] Ratios lower than 1.00 indicate increased specificity for $\alpha$-(1,4) over $\alpha$-(1,6)-linked substrates.

TABLE 15

Increase in the optimum pH of the enzyme-substrate complex of mutant glucoamylases for hydrolysis of maltose at 45°C compared to that of wild-type.

| GA form | $pH_{opt}$ Increase[a] |
|---------|------------------------|
| S411G | 0.26 |
| S411A | 0.84 |
| S411C | 0.86 |
| S411H | 0.15 |
| S411D | 0.46 |

[a]The pH optimum of the enzyme-substrate complex of wildtype glucoamylase for hydrolysis of maltose at 45°C was pH 3.98.

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Williamson, et al (1992) *Biochem. J.*, **282**, 423-428.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Allen, Martin
                 Fang, Tsuei-Yun
                 Li, Yuxing
                 Liu, Hsuan-Liang
                 Chen, Hsui-Mei
                 Coutinho, Pedro
                 Hanzatko, Richard
                 Ford, Clark

    (ii) TITLE OF INVENTION: PROTEIN ENGINEERING OF GLUCOAMYLASE TO
         INCREASE pH OPTIMUM, SUBSTRATE SPECIFICITY AND
         THERMOSTABILITY

   (iii) NUMBER OF SEQUENCES: 12

    (iv) CORRESPONDENCE ADDRESS:
         (A) ADDRESSEE: Kohn & Associates
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         (F) ZIP: 48334

    (v) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
         (B) COMPUTER: IBM PC compatible
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
         (A) APPLICATION NUMBER:
         (B) FILING DATE:
         (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
         (A) NAME: Kohn, Kenneth I.
         (B) REGISTRATION NUMBER: 30,955
         (C) REFERENCE/DOCKET NUMBER: 0812.00001

    (ix) TELECOMMUNICATION INFORMATION:
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         (B) TELEFAX: (248) 539-5055


(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 616 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

   (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
         (A) ORGANISM: Aspergillus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ala Thr Leu Asp Ser Trp Leu Ser Asn Glu Ala Thr Val Ala Arg Thr
1               5                   10                  15

Ala Ile Leu Asn Asn Ile Gly Ala Asp Gly Ala Trp Val Ser Gly Ala
            20                  25                  30

Asp Ser Gly Ile Val Val Ala Ser Pro Ser Thr Asp Asn Pro Asp Tyr
        35                  40                  45

Phe Tyr Thr Trp Thr Arg Asp Ser Gly Leu Val Leu Lys Thr Leu Val
    50                  55                  60

Asp Leu Phe Arg Asn Gly Asp Thr Ser Leu Leu Ser Thr Ile Glu Asn
65                  70                  75                  80

Tyr Ile Ser Ala Gln Ala Ile Val Gln Gly Ile Ser Asn Pro Ser Gly
                85                  90                  95

Asp Leu Ser Ser Gly Ala Gly Leu Gly Glu Pro Lys Phe Asn Val Asp
            100                 105                 110

Glu Thr Ala Tyr Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp Gly Pro
    115                 120                 125

Ala Leu Arg Ala Thr Ala Met Ile Gly Phe Gly Gln Trp Leu Leu Asp
    130                 135                 140

Asn Gly Tyr Thr Ser Thr Ala Thr Asp Ile Val Trp Pro Leu Val Arg
145                 150                 155                 160

Asn Asp Leu Ser Tyr Val Ala Gln Tyr Trp Asn Gln Thr Gly Tyr Asp
            165                 170                 175

Leu Trp Glu Glu Val Asn Gly Ser Ser Phe Phe Thr Ile Ala Val Gln
        180                 185                 190

His Arg Ala Leu Val Glu Gly Ser Ala Phe Ala Thr Ala Val Gly Ser
    195                 200                 205

Ser Cys Ser Trp Cys Asp Ser Gln Ala Pro Glu Ile Leu Cys Tyr Leu
    210                 215                 220

Gln Ser Phe Trp Thr Gly Ser Phe Ile Leu Ala Asn Phe Asp Ser Ser
225                 230                 235                 240

Arg Ser Gly Lys Asp Ala Asn Thr Leu Leu Gly Ser Ile His Thr Phe
            245                 250                 255

Asp Pro Glu Ala Ala Cys Asp Asp Ser Thr Phe Gln Pro Cys Ser Pro
        260                 265                 270

Arg Ala Leu Ala Asn His Lys Glu Val Val Asp Ser Phe Arg Ser Ile
        275                 280                 285

Tyr Thr Leu Asn Asp Gly Leu Ser Asp Ser Glu Ala Val Ala Val Gly
    290                 295                 300

Arg Tyr Pro Glu Asp Thr Tyr Tyr Asn Gly Asn Pro Trp Phe Leu Cys
305                 310                 315                 320

Thr Leu Ala Ala Ala Glu Gln Leu Tyr Asp Ala Leu Tyr Gln Trp Asp
                325                 330                 335
```

```
Lys Gln Gly Ser Leu Glu Val Thr Asp Val Ser Leu Asp Phe Phe Lys
        340             345             350

Ala Leu Tyr Ser Asp Ala Ala Thr Gly Thr Tyr Ser Ser Ser Ser Ser
        355             360             365

Thr Tyr Ser Ser Ile Val Asp Ala Val Lys Thr Phe Ala Asp Gly Phe
    370             375             380

Val Ser Ile Val Glu Thr His Ala Ala Ser Asn Gly Ser Met Ser Glu
385             390             395             400

Gln Tyr Asp Lys Ser Asp Gly Glu Gln Leu Ser Ala Arg Asp Leu Thr
            405             410             415

Trp Ser Tyr Ala Ala Leu Leu Thr Ala Asn Asn Arg Arg Asn Ser Val
            420             425             430

Val Pro Ala Ser Trp Gly Glu Thr Ser Ala Ser Ser Val Pro Gly Thr
        435             440             445

Cys Ala Ala Thr Ser Ala Ile Gly Thr Tyr Ser Ser Val Thr Val Thr
    450             455             460

Ser Trp Pro Ser Ile Val Ala Thr Gly Gly Thr Thr Thr Ala Thr
465             470             475             ,   480

Pro Thr Gly Ser Gly Ser Val Thr Ser Thr Ser Lys Thr Thr Ala Thr
            485             490             495

Ala Ser Lys Thr Ser Thr Ser Thr Ser Ser Thr Ser Cys Thr Thr Pro
            500             505             510

Thr Ala Val Ala Val Thr Phe Asp Leu Thr Ala Thr Thr Thr Tyr Gly
        515             520             525

Glu Asn Ile Tyr Leu Val Gly Ser Ile Ser Gln Leu Gly Asp Trp Glu
    530             535             540

Thr Ser Asp Gly Ile Ala Leu Ser Ala Asp Lys Tyr Thr Ser Ser Asp
545             550             555             560

Pro Leu Trp Tyr Val Thr Val Thr Leu Pro Ala Gly Glu Ser Phe Glu
            565             570             575

Tyr Lys Phe Ile Arg Ile Glu Ser Asp Asp Ser Val Glu Trp Glu Ser
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(2) INFORMATION FOR SEQ ID NO:2:

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        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

-78-


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

        Asn Gly Asn Gly Asn Ser Gln
        1                   5

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
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            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"



        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGAGTCCGC GCCCGGCACC CAAGCACCGT C                                    31

(2) INFORMATION FOR SEQ ID NO:4:

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            (A) LENGTH: 33 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"



        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAGTCCAGCG ACACAGGTGT GACCTCCAAC GAC                                  33

(2) INFORMATION FOR SEQ ID NO:5:

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            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"



        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGAGCGGAAA GCTGCGGGCC ATCAGACTTG TC                                   32

(2) INFORMATION FOR SEQ ID NO:6:

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            (A) LENGTH: 32 base pairs
            (B) TYPE: nucleic acid

-79-

```
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"
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       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGTACTGCCA TCCTGTGTAA CATCGGGGCG GA                              32

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            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"
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       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCGGGGCGG ACGGTTGTTG GGTGTCGGGC GCG                             33

(2) INFORMATION FOR SEQ ID NO:8:

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            (A) LENGTH: 25 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"
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GAGTATCGTG TGTACTGGCG GCACC                                     25

(2) INFORMATION FOR SEQ ID NO:9:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
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       (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "PRIMER"
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       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
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-80-


GGTCTCGGTG AGCCCAGGTT CAATGTCGAT                                           30

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "PRIMER"



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GGTCTCGGTG AGCCCATGTT CAATGTCGAT                                           30

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "PRIMER"



        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GAGGACACGT ACTGGAACGG CAACCCG                                              27

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 60 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
             (A) DESCRIPTION: /desc = "PRIMER"



        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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## CLAIMS

What is claimed is:

1.  A fungal glucoamylase including a mutation pair Asn20Cys coupled with Ala27Cys forming a disulfide bond between the two members of the pair.

2.  The glucoamylase as set forth in claim 1 wherein the mutation provides increased thermal stability and reduced isomaltose formation.

3.  The fungal glucoamylase as set forth in claim 1 and including at least one mutations selected from Table 13 wherein cumulative thermal stability is provided by the additional mutations.

4.  The fungal glucoamylase as set forth in claim 1 further including mutations Ser30Pro, Gly137Ala wherein cumulative thermal stability is provided by the additional mutations.

5.  The fungal glucoamylase as set forth in claim 1 and including at least one mutation from Table 14 wherein cumulative reduced isomaltose formation is provided by the additional mutations.

6.  The fungal glucoamylase as set forth in claim 1 further including a 311-314Loop mutation wherein cumulative reduced isomaltose formation is provided by the mutation.

7.  A fungal glucoamylase including a 311-314Loop mutation.

8.  The glucoamylase as set forth in claim 7

wherein reduced isomaltose formation is provided by the
mutation.


9.    The fungal glucoamylase as set forth in claim
7 and including at least one mutation from Table 14
wherein cumulative reduced isomaltose formation is
provided by the additional mutation.


10.   A fungal glucoamylase including a mutation
Ser411Ala.


11.   The glucoamylase as set forth in claim 10
wherein increased pH optimum and reduced isomaltose
formation is provided by the mutation.


12.   The fungal glucoamylase as set forth in claim
10 and including at least one mutation from Table 15
wherein cumulative increased pH optimum is provided by
the mutations.


13.   The fungal glucoamylase as set forth in claim
10 and including at least one mutation from Table 14
wherein cumulative reduced isomaltose formation is
provided by the mutations.


14.   A fungal glucoamylase including a Ser411Ala
mutation and a mutation pair Asn20Cys coupled with
Ala27Cys forming a disulfide bond between the two
members of the pair.


15.   The glucoamylase as set forth in claim 14
wherein increased thermal stability, increased pH
optimum and reduced isomaltose formation are provided
by the mutations.


16.   A fungal glucoamylase including a Ser411Ala

mutation and a mutation pair Asn20Cys coupled with
Ala27Cys forming a disulfide bond between the two
members of the pair and a 311-314Loop mutation.

17.   The glucoamylase as set forth in claim 16
wherein increased thermal stability, increased pH
optimum and reduced isomaltose formation are provided
by the mutations.

18.   A vector containing the cDNA for an
engineered glucoamylase as set forth in claims 1-17.

19.   A host cell transformed with the vector of
claim 18.

20.   A fungal glucoamylase as set forth in claims
1-17 wherein the glucoamylase is an *Aspergillus*
glucoamylase.

21.   The glucoamylase as set forth in claim 20
wherein the glucoamylase is *Aspergillus awamori*
glucoamylase.

22.   A method to obtain a fungal glucoamylase with
decreased thermal inactivation by designing mutations
having decreased conformational entropy of unfolding,
or increased stability of α-helices, or increased
disulfide bonds or hydrogen bonding and electrostatic
interactions and hydrophic interactions and Vanderwalls
interactions and packing compactness.

23.   A method to obtain a fungal glucoamylase with
reduced isomaltose formation by designing mutations to
decrease the α-(1,6)-glucosidic linkage affinity.

24.   A method to obtain a fungal glucoamylase with

increased pH optimum by changing the polarity, charge
distribution and hydrogen bonding in the
microenvironment of the catalytic base Glu400.


25. A method of selecting mutations for fungal
glucoamylase to be used for constructing glucoamylases
with cumulative mutations by

designing and generating individual mutations by
site directed mutagenesis;

screening the individual mutations and selecting
those which show at least increased pH optimum,
decreased irreversible thermal inactivation rates or
reduced isomaltose formation;

performing site directed mutagenesis to produce
enzymes carrying at least two of the isolated selected
mutations for either increased pH optimum, decreased
irreversible thermal inactivation rates or reduced
isomaltose formation; and

screening for cumulatively additive effects of the
mutations on pH optimum, thermal stabilizing or reduced
isomaltose formation by the produced enzymes carrying
at least two of the isolated selected mutations.

Fig-1



Fig-2

*Fig-3*

_Fig-4_

Fig-5A



Fig-5B
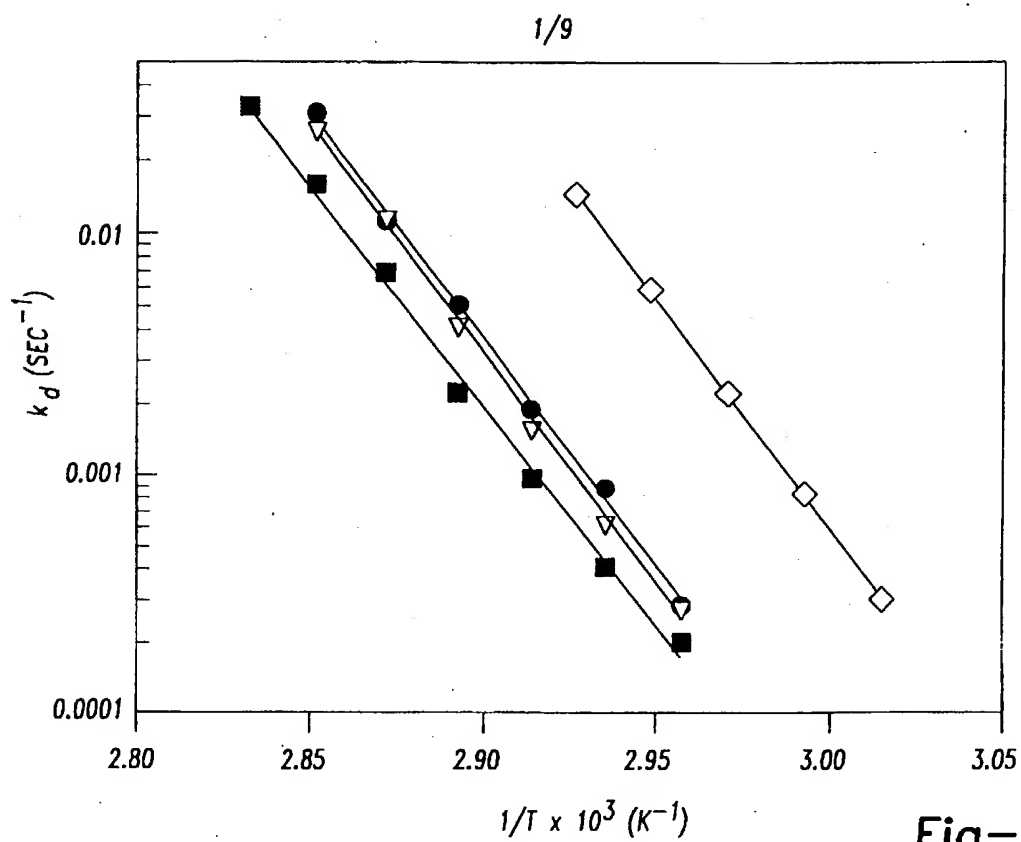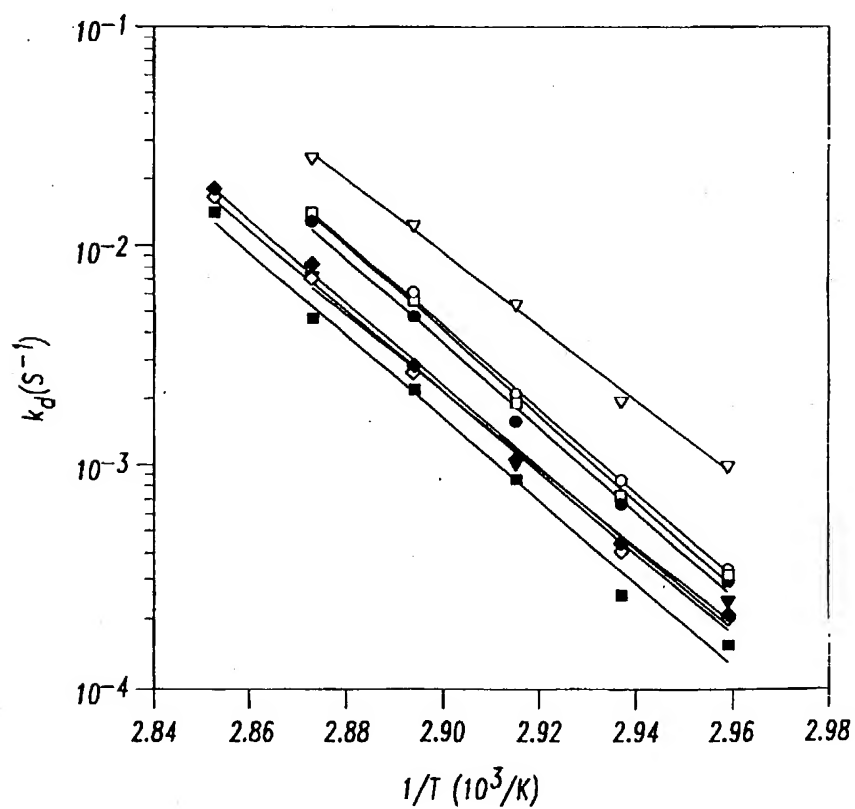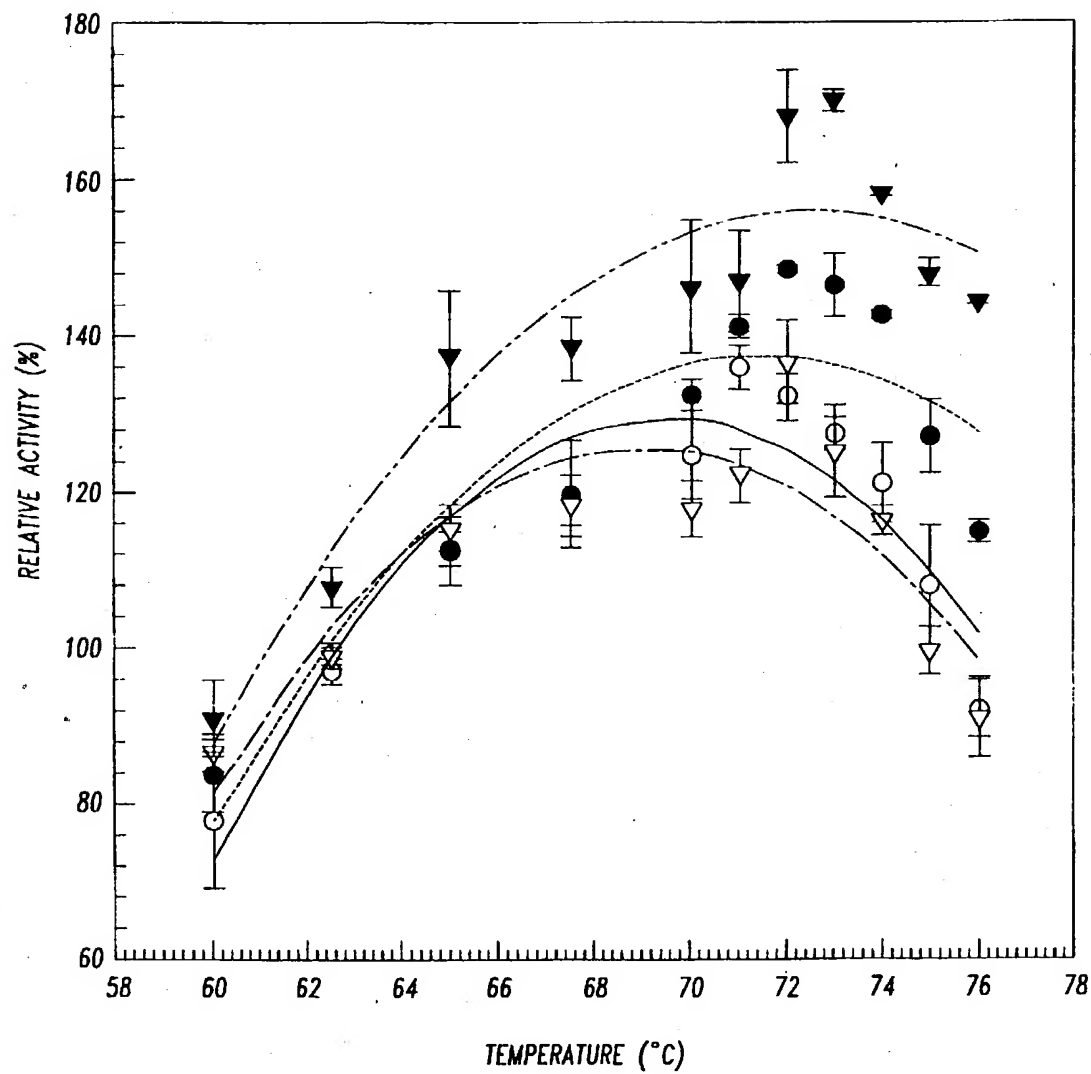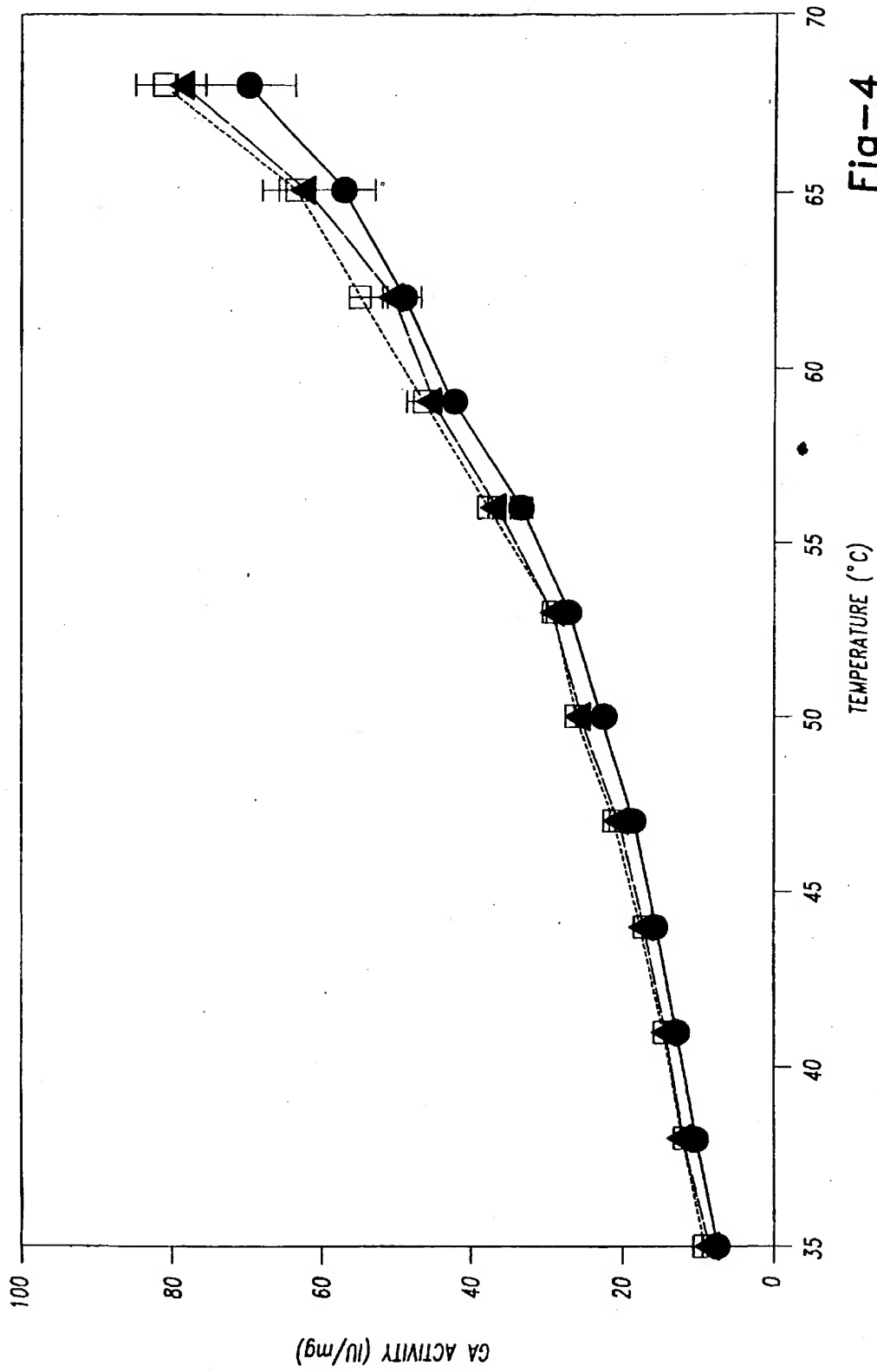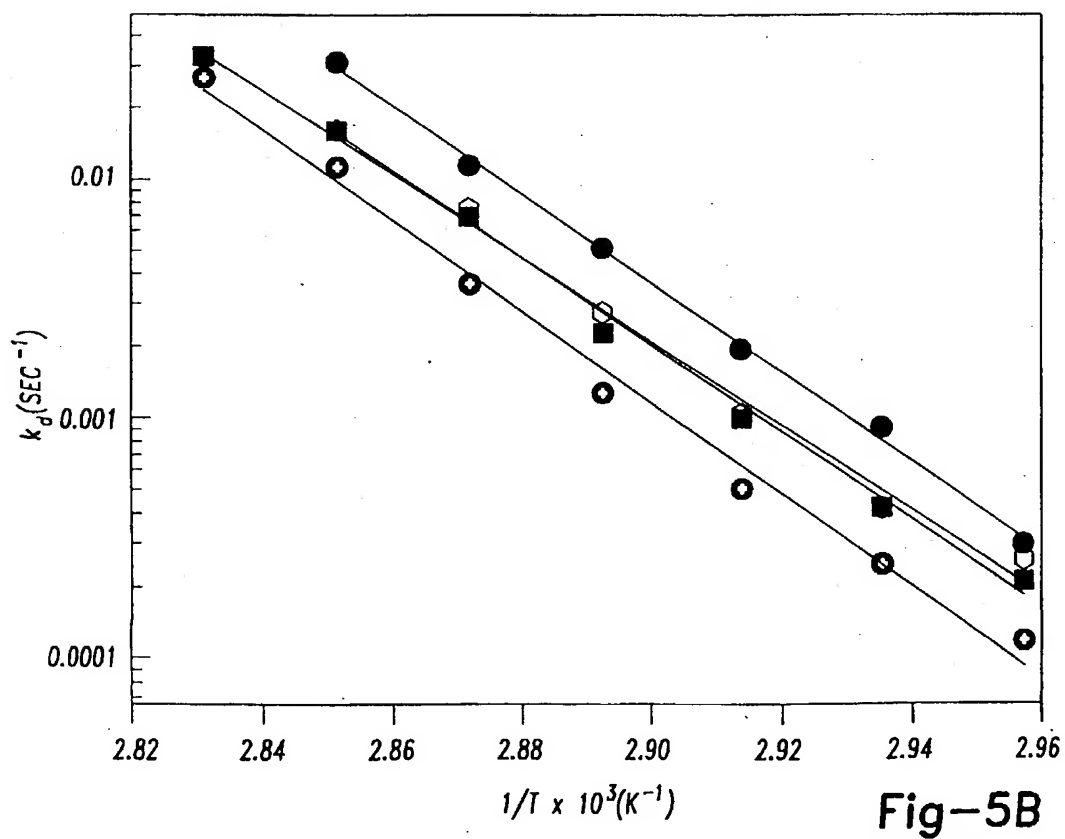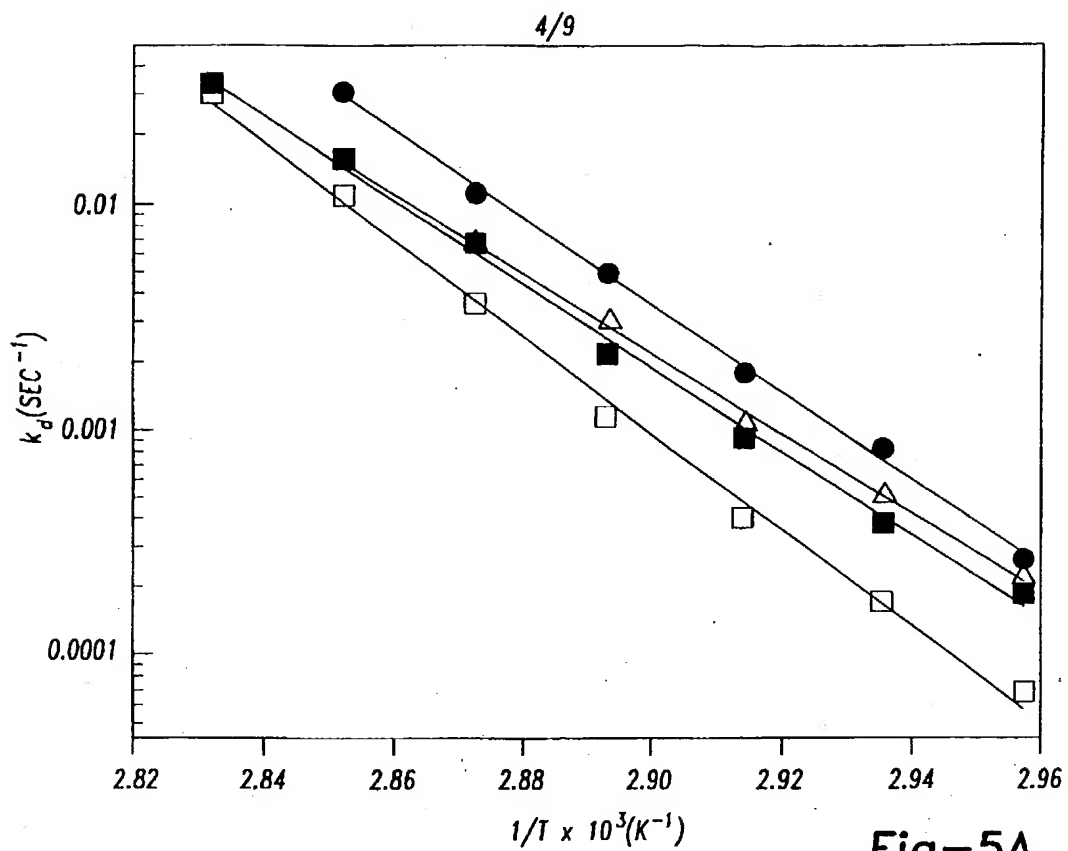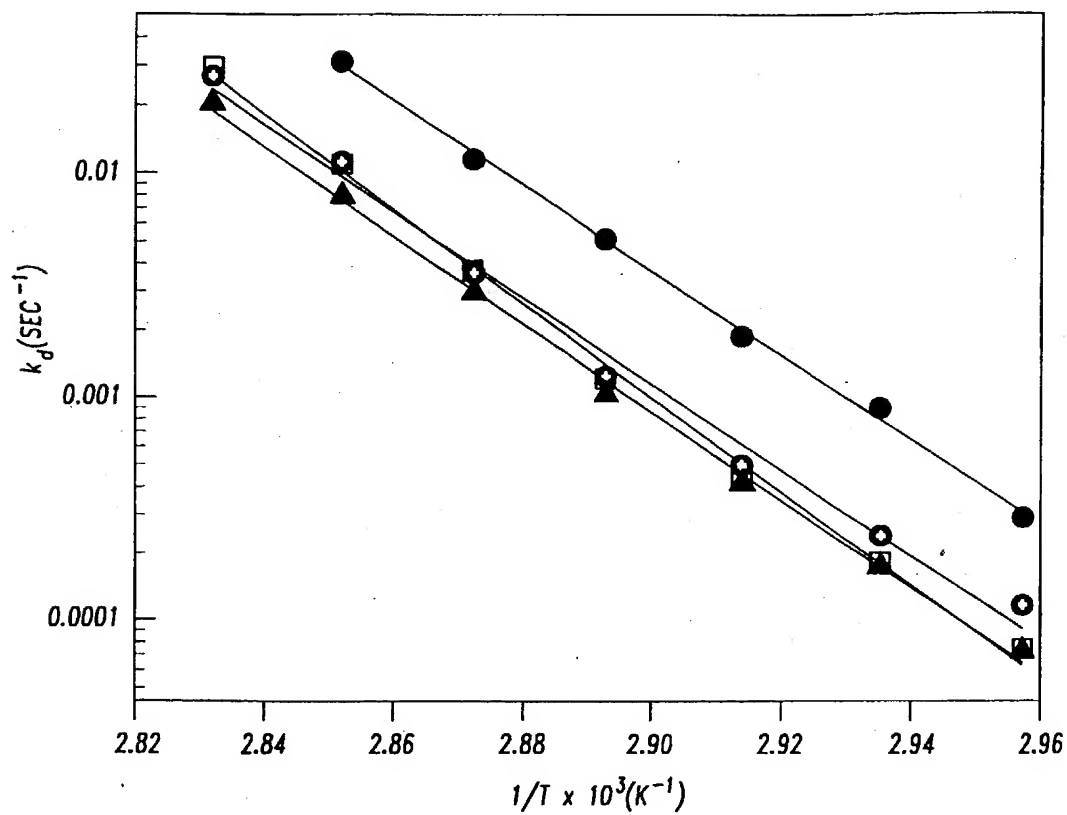
## Fig-5C

55°C



Fig—6A

65°C
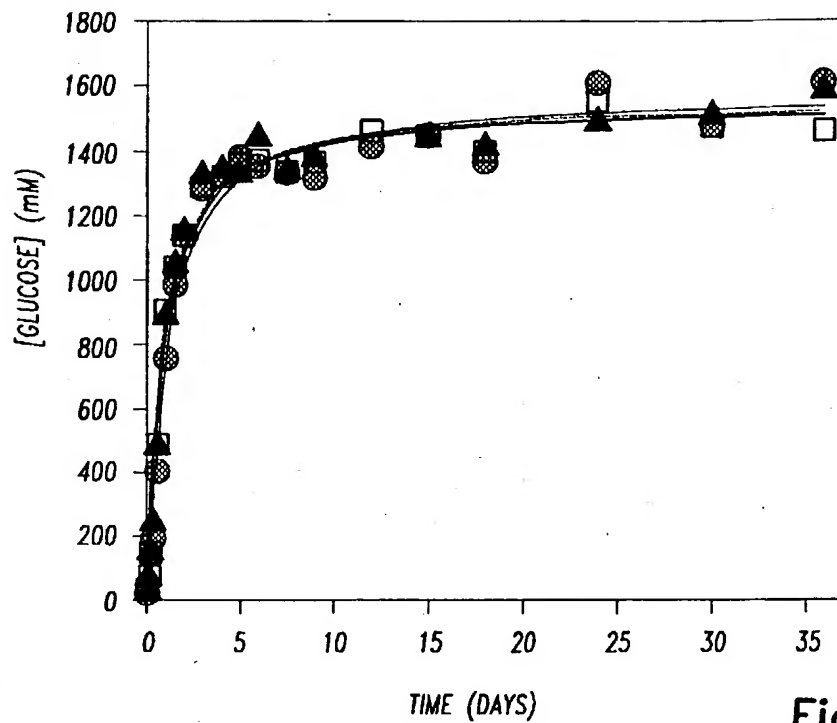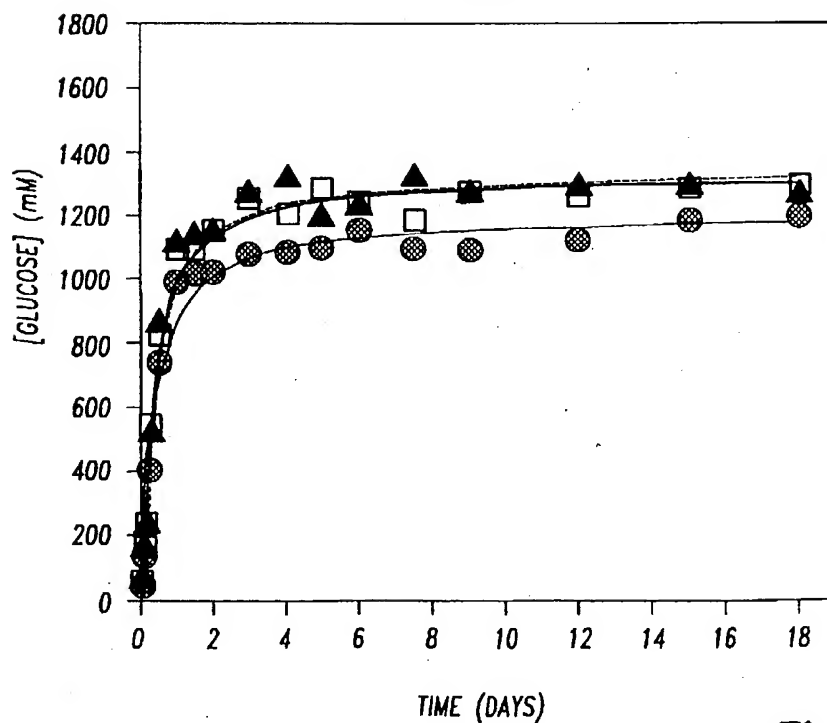


Fig—6B

Fig-7
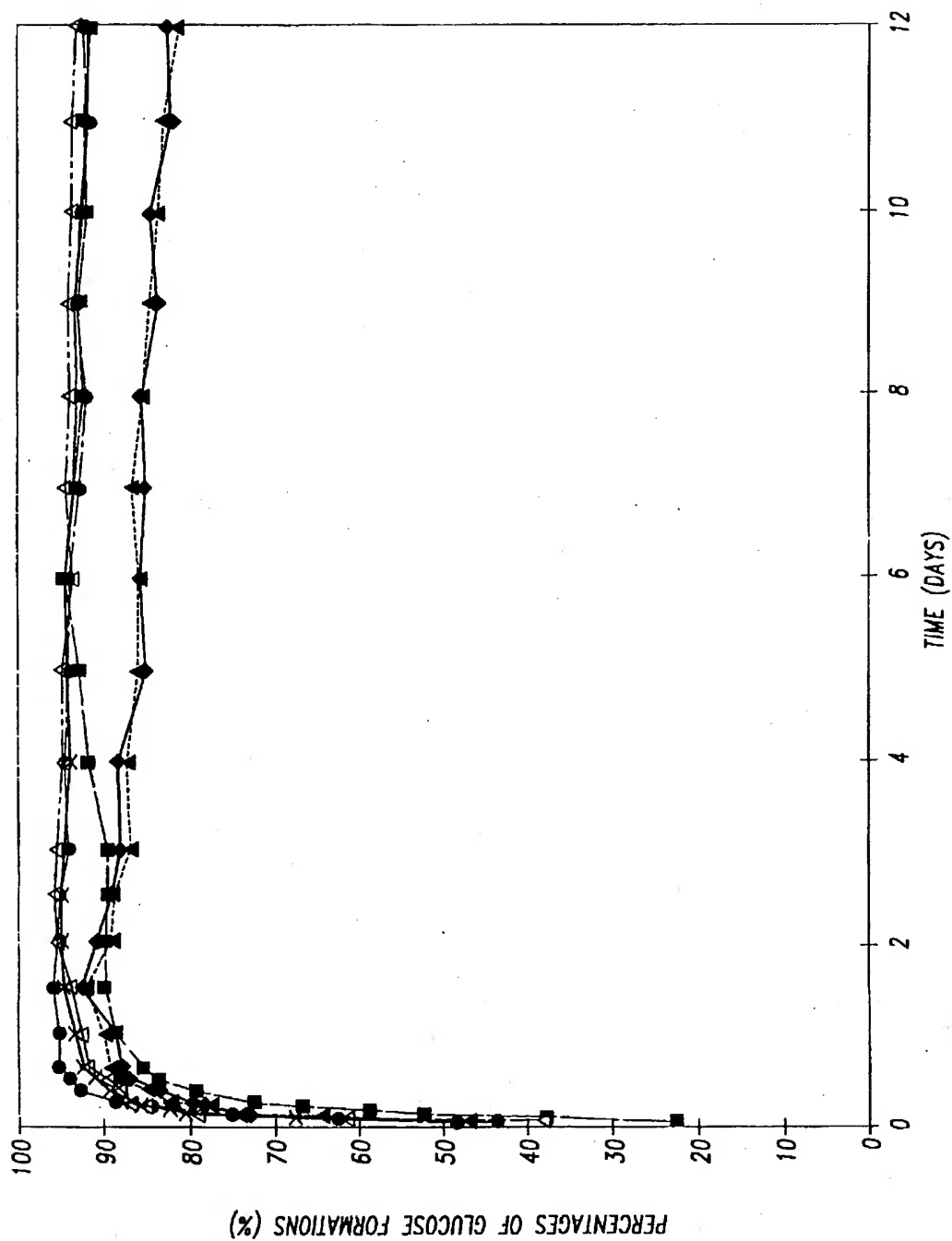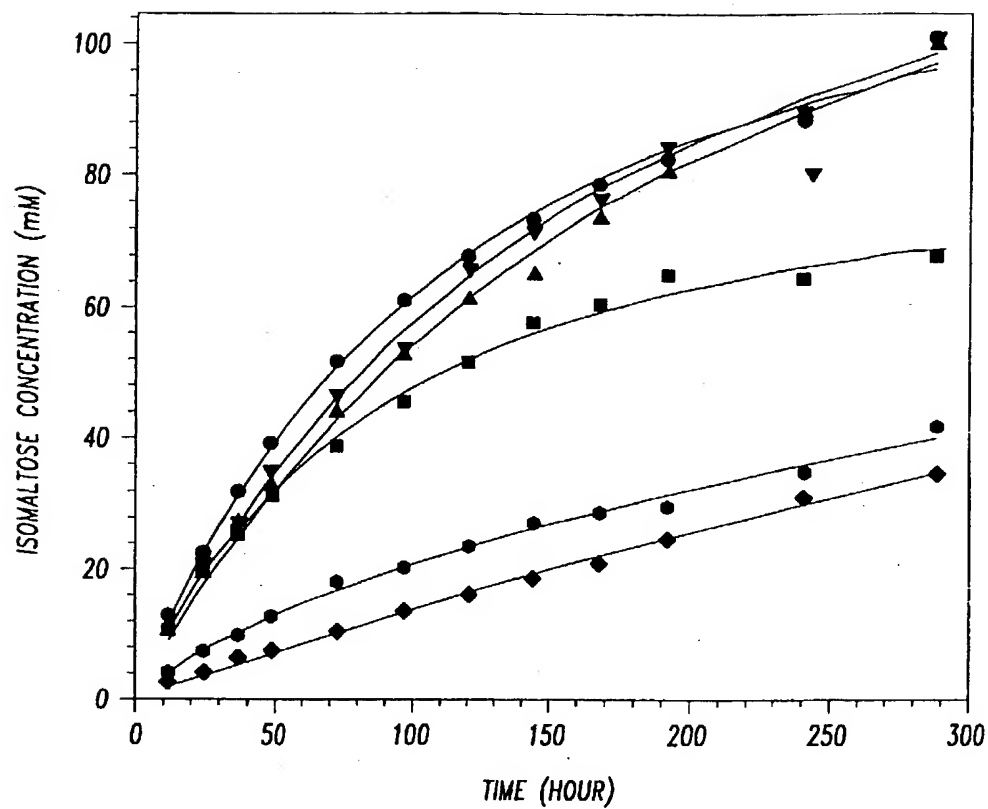
## Fig-8
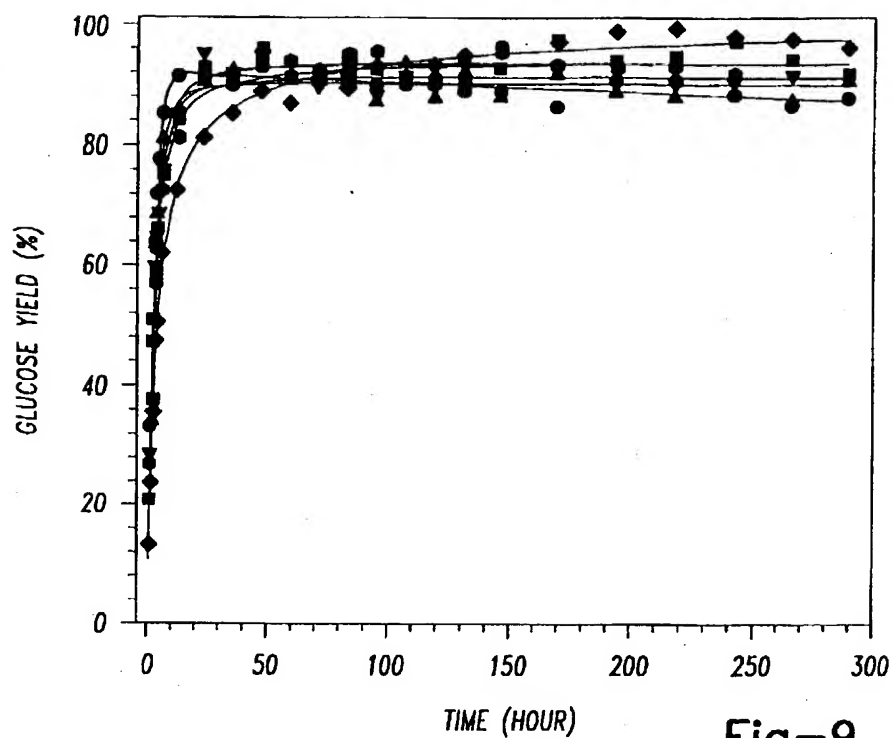
Fig-9
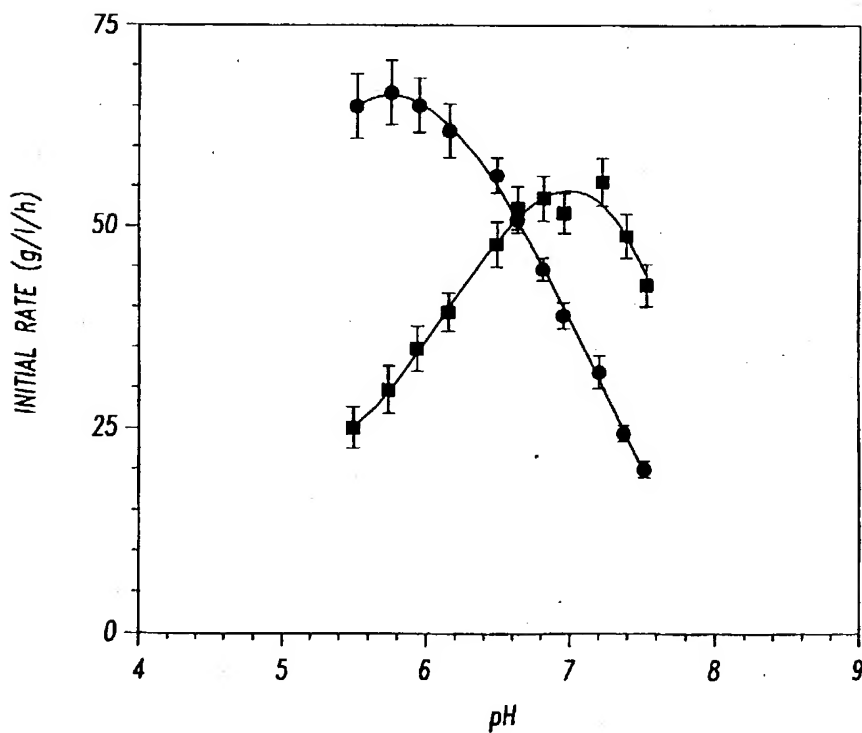


Fig-10

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12983

| A. CLASSIFICATION OF SUBJECT MATTER |
|---|
| IPC(6)  :C12N 9/34, 1/21, 15/63, 15/09 |
| US CL  :435/205, 252.3, 320.1, 172.1 |
| According to International Patent Classification (IPC) or to both national classification and IPC |

| B. FIELDS SEARCHED |
|---|
| Minimum documentation searched (classification system followed by classification symbols) |
| U.S. :  435/205, 252.3, 320.1, 172.1 |

| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched |
|---|
|  |

| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) |
|---|
| Please See Extra Sheet. |

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X,P<br>—<br>Y,P | Li. Genetic construction and biochemical analysis of thermostability mutants of glucoamylase from aspergillus awamori. Dissertation Abstracts International. Abstract No. 97:24090, 1996, Vol. 57, No. 11B, page 6761, see abstract. | 1, 2, 22<br>------------<br>3-6, 25 |
| X<br>---<br>Y | CHEN. Site-directed mutagenesis to enhance thermostability of Aspergillus awamori glucoamylase expressed in Saccharomyces cerevisiae. Dissertation Abstracts International. Abstract No. 95(03):B0023, 1994, Vol. 54, page 5998, see abstract. | 22<br>------------<br>3-5, 9, 13 |

| [X] | Further documents are listed in the continuation of Box C. | [ ] | See patent family annex. |
|---|---|---|---|

| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
|---|---|---|---|
| "A" | document defining the general state of the art which is not considered to be of particular relevance | | |
| "E" | earlier document published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 30 SEPTEMBER 1997 | 31 OCT 1997 |

| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer  *[signature]*<br>KAWAI LAU |
|---|---|
| Facsimile No.    (703) 305-3230 | Telephone No.    (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X<br>---<br>Y | CHEN et al. Effect of replacing helical glycine residues with alanines on reversible and irreversible stability and production of Aspergillus awamori glucoamylase. Protein Engineering. June 1996, Vol. 9, No. 6, pages 499-505, see whole document. | 22<br>------------<br>3-5, 9, 13 |
| Y,P | STOFFER et al. Glucoamylase mutants in the conserved active-site segment Trp170-Tyr175 located at a distance from the site of catalysis. Protein Engineering. January 1997, Vol. 10, No. 1, pages 81-87, see whole document. | 5, 9, 13 |
| X<br>---<br>Y | FIEROBE et al. Mutational modulation of substrate bond-type specificity and thermostability of glucoamylase from Aspergillus awamori by replacement with short homologue active site sequences and thiol/disulfide engineering. Biochemistry. 02 July 1996, Vol. 35, No. 26, pages 8696-8704, see whole document. | 22<br>---<br>25 |
| X<br>---<br>Y | SIERCKS et al. Protein engineering of the relative specificity of glucoamylase from Aspergillus awamori based on sequence similarities between starch-degrading enzymes. Protein Engineering. December 1994, Vol. 7, No. 12, pages 1479-1484, see whole document. | 23<br>---<br>25 |
| X<br>---<br>Y | BAKIR et al. Cassette mutagenesis of the active site of Aspergillus awamori glucoamylase. Protein Engineering. Abstract No. 93-04739, March 1993, Vol. 6, Suppl., page 41, see abstract. | 24<br>---<br>25 |

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN-USpatfull, CJACS, Europatfull, Biotechds, CAPlus, CJElsevier, Medline, Biosis, Scisearch, Toxline, JICST-EPlus, Embase, FSTA, Agricola, Cancerlit, Ifipat, CEABA, Lifesci, Toxlit, Prompt, CABA, Dissabs, WPIDS, Biobusiness, Paperchem2, Aquasci, CEN, Japio, Patosep

search terms: glucoamylase, mutant, mutation, mutagenesis, variant, modify, alter, homolog, analog, mutein, substitute, thermostability, isomaltose, pH optimum